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EVALUATION OF THE EXPRESSION OF MATRIX METALLOPROTEINASE 9 AND NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN IN SERUM, URINE AND TUMORAL TISSUES OF FEMALE DOGS SUFFERING FROM MAMMARY GLAND TUMORS

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ABBREVIATIONS

AUC: area under curve

BMT: benign mammary tumor

BSA: bovine serum albumin

DAB: 3,3'-diaminobenzidine

ddH2O: double distilled water

DEPC: diethylpyrocarbonate

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FS: final score (for immunohistochemistry scoring)

IHC: immunohistochemistry

IMAC: immobilized metal ion affinity chromatography

IPTG: isopropylthio-β-galactoside

IS: intensity score (for immunohistochemistry scoring)

LB: lysogeny broth

MGT: mammary gland tumor

MMP-2: matrix metalloproteinase 2

MMP-9: matrix metalloproteinase 9

MMP-9-Dist: distal part of MMP-9

MMP-9-Prox: proximal part of MMP-9

NGAL: neutrophil gelatinase-associated lipocalin

MMT: malignant mammary tumor

OD: optical density

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline tween-20

PCR: polymerase chain reaction

PS: proportion score (for immunohistochemistry scoring)

RT: Reverse transcription

TAE: tris acetate EDTA

TIMPs: tissue inhibitors of metalloproteinases

TMB: tétraméthylbenzidine

INTRODUCTION

Mammary gland tumors are the most common type of tumors in female dogs, with an incidence higher than 25% in unsprayed females. This incidence is more than three times higher than the one of breast cancer in human medicine. Constant research projects are lead to find new ways to diagnose, evaluate, follow-up and treat this pathology in both species.

Matrix metalloproteinase 9 (MMP-9), also known as gelatinase B, is a major contributor to the proteolytic degradation of extracellular matrix during tumor invasion. In human breast cancer research studies, this protein was shown to be highly expressed in the serum, the blood and the mammary gland histological tissue of breast cancer patients and several authors have suggested the use of MMP-9 as a biomarker of breast cancer.

Neutrophil gelatinase-associated lipocalin (NGAL) was first isolated in 1992 as a small glycoprotein bound with a covalent link to MMP-9 in human neutrophils. It was later showed that NGAL can protect MMP-9 from degradation and that it was also a valuable biomarker of breast cancer disease in the urine, serum and mammary tissue of human patients.

Very few research projects in veterinary medicine have aimed to investigate the correlation between canine mammary gland tumor and the two proteins. Only few studies have reported the use of anti human-MMP-9 antibodies in immunohistochemical staining of canine mammary gland tissue and it was demonstrated that its expression was significantly correlated with several prognostic factors such as malignancy status, histological grade, lymph node metastases and lower disease-free survival. No report has been made concerning NGAL and its correlation with any canine tumor.

After a short bibliographic review on the findings made concerning NGAL and MMP-9 in both human and veterinary medicine, the aim of this study was to use canine specific anti - NGAL and anti MMP-9 antibodies to evaluate the expression of the two proteins in different biologic samples from patients suffering from at least one mammary tumor. Blood serum and urinary NGAL expression was analyzed by enzyme-linked immunosorbent assay (ELISA) while both NGAL and MMP-9 expression in mammary gland tissue sections was evaluated by immunohistochemistry (IHC) after formalin fixation.

This study was performed at the Microbiology and Public Health Department of Chung-Hsing University in Taichung, Taiwan.

CURRENT KNOWLEDGE ON MATRIX-METALLOPROTEINASE-9 AND NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN: REVIEW

1. MATRIX METALLOPROTEINASE 9

1.1. Introduction to MMPs

The extracellular matrix is constantly degraded, repaired and remodeled and this phenomenon is essential for the embryonic development, the morphogenesis or the tissue repairs. Several types of proteinases play an important role in the degradation of this extracellular matrix but the major ones are believed to be the matrix metalloproteinases, also called MMPs (Visse et al.,2003).

Humans have 23 different MMPs but the activity of most of them is very low in normal tissues. Their expression is under the influence of many factors such as inflammatory cytokines, growth factors, hormones or cell-matrix. All MMPs are secreted as inactive pro-enzymes. Their proteolytic activities are precisely regulated by the activation from the precursor and by inhibition with endogenous inhibitors, mainly tissue inhibitors of metalloproteinases, also known as TIMPs and α2-macroglobulin (Nagase et al., 1999).

1.2. MMP-9 general structure

Most MMPs, including MMP-9, contain a propeptide, a catalytic metalloproteinase domain with a zinc binding motif, a hinge region (linker), and a hemopexin domain. The arrangement of those typical domains, the presence of other domains and the substrate preferences lead to the separation of the MMPs in different groups such as collagenases, gelatinases, matrilysins and others.

Gelatinases, which include MMP-2, known as gelatinase A, and MMP-9, known as gelatinase B, can digest a large number of extracellular matrix molecules such as gelatin, type IV, V and XI collagens, elastin, laminin, aggrecan core proteins (Nagase et al., 2006). They are recognized by the three repeats of a fibronectin type II motif they have in the middle of their catalytic domain (figure 1). This particular domain helps them to bind the gelatin or collagen in order to digest it. Unlike MMP-2, MMP-9 is not able to digest type I, II and III collagens.

The role of the hinge region is still unclear, but the hemopexin domain seems to mediate many inter-protein interactions. It plays a role in the binding of the TIMPs, in the activation of the protein, in the binding of some substrates and also in the homodimerization of MMP-9 (Cha et al., 2002).

Human MMP-9 is constituted by 707 amino-acid residues while canine MMP-9's sequence is 3 amino-acids shorter (NCBI Protein Data Bank).

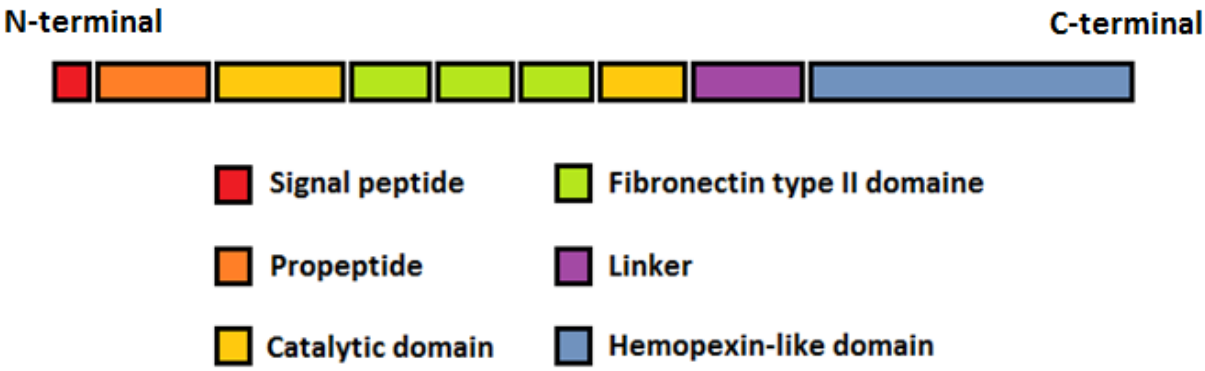


Figure 1a: The different domains constituting MMP-9

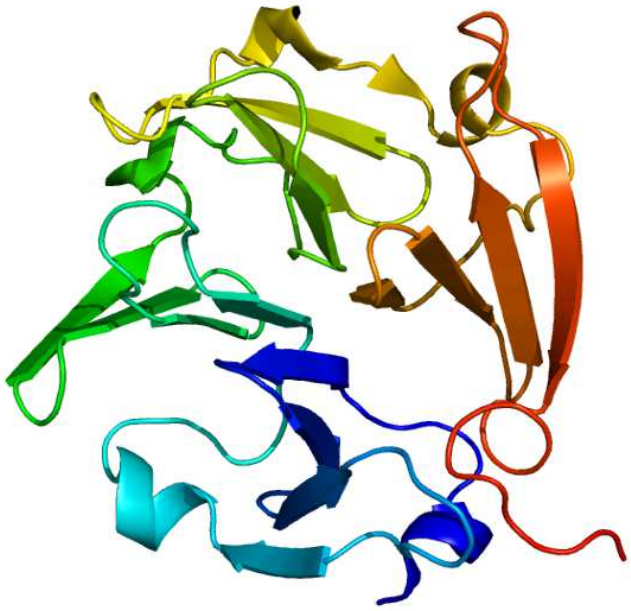


Figure 1b: NCBI Protein Data Bank (PDB) rendering of human MMP-9

Figure 1: Architectural organization of human MMP-9

1.3. Regulation of MMP-9 activity

In order to avoid undesired matrix damage, the activity of such proteins has to be accurately controlled. This control is mainly mediated by the activation of the pro-enzyme and the inhibition of the active enzyme by endogenous inhibitors.

- Activation of pro enzyme

MMP-9 is synthesized as a pro-enzyme. The signal peptide is removed during the translation step and pro-MMP-9 is secreted, mainly from inflammatory cells such as neutrophils, monocytes/macrophages and mast cells but lower expression intensity can be found in epithelial and endothelial cells, fibroblasts and sometimes hematopoietic cells. However, this pro-enzyme is inactive. At one end of the pro-MMP-9 is the pro-peptide domain which allows the enzyme to be active after it has been cleaved by a complicated activation network cells (Opdenakker et al., 2002). It would seem that the pro-MMP-9 can also be activated by other mechanisms: for instance, neutrophils have been shown to activate the enzyme via an oxidative mechanism (Peppin et al., 1986). Overall, all the mechanisms that lie behind the activation of MMP-9 are still unclear and it must be noted that some findings also revealed that the pro-MMP-9 could in some cases display significant catalytic activity in the presence of the propeptide (Bannikov et al., 2002).

- Inhibitors of MMP activity

Many naturally occurring gelatinase inhibitors have been studied. The most important ones are mainly α2-magroglobulin and tissue inhibitor of metalloproteinase also known as TIMPs. α2-macroglobulin is a relatively large protein that is abundant in the serum and is able to bind to the active form of the enzyme (Morodomi et al., 1992). TIMPs form a large family of small proteins, containing around 170-180 amino acids, that can form a high-affinity complex with metalloproteinase and in particular with MMP-9 (Egeblad et al., 2002). The complex formed by the inhibitor and the MMP-9 are internalized into the cells and is degraded.
Unlike the other collagenase MMP-2, MMP-9 expression is highly inducible under the influence of many factors such as growth factors, chemokines, and a wide array of other stimulatory signals such as integrin or extracellular matrix-mediated signals (Hipps et al., 1991).

1.4. Physiological roles of MMP-9

MMP-9 plays a wide array of roles in many different physiological processes such as growth and development, immune modulation, inflammatory status, wound healing and several more. MMP-9 was shown to play very important physiological roles as early as in the embryonic development stages. It was shown to be highly expressed in the mouse embryo (Alexander et al., 1996). However, MMP-9 knocked out mice were viable but showed developmental abnormalities. Its main effects have been noticed in particular on the vascularization and ossification as the knockout resulted in skeletal abnormalities, mainly a delayed ossification of the growth plate in long bones as MMP-9 was shown to be a key regulator of growth plate angiogenesis and apoptosis of chondrocytes (Vu et al., 1998). Although MMP-9 is highly expressed in immune cells such as neutrophils, no important immune-related abnormality was reported in the knocked-out mice.

Reproduction is also a domain in which MMP-9 seems to be important. In particular, it has been suggested to be involved at different phases of the cyclic changes in female reproduction, mainly in the menstrual cycle (mostly the matrix remodeling during the cycle), the ovulation, the embryo implantation, the parturition (degradation of the extracellular matrix of the fetal membranes to facilitate their rupture), and even the involution of the mammary glands after the lactation (Vadillo-Ortega et al., 1995; Jeziorska et al., 1996). MMP-9 deficiency was also proven to impair reproduction as Dubois and coworkers showed that MMP-9 knockout mice led to a higher percentage of infertile breeding pairs, a smaller number of litters and a smaller size of the litters (Dubois et al., 2000).

Leukocytes need to adhere to the endothelium of the blood-vessels to reach the targeted tissue. They use large amounts of protease-containing granules, which are rapidly delivered to the cell surface and secreted into the extracellular milieu. One of these granules was named gelatinase granule as it contained a high amount of MMP-9 (Kjeldsen et al., 1994).

MMP-9 plays an important role in wound healing and in coordinating epithelial regeneration in general. For instance, it was shown *in vitro* that the keratinocyte migration was dependent on induction of MMP-9 and is impacted by blocking the protein with a specific antibody or with a general MMP inhibitor (McCawley et al., 1998). The role of MMP-9 in wound healing is yet unclear as it participates with several other MMPs in the inflammation stage (regulation of chemokine activity, establishment of chemotactic gradients, extravasation of leukocytes into the injured tissue…) and the epithelial repair stage of wound healing (Gill et al., 2008).
MMP-9 was also shown to play significant roles in angiogenic revascularization of ischemic tissues (Johnson et al., 2004), or remyelination (Larsen et al., 2003).

MMP-9 shows a wide physiological function panel but an increase of its activity has also been reported in several pathological status, such as cancer, inflammatory or infectious diseases, vascular diseases and many more (Van den Steen et al., 2002), but the most important pathological roles it plays are most certainly in tumor progression.

1.5. MMP-9 in tumor progression

To enter and exit vasculature at the primary and metastatic sites, metastatic tumor cells must cross the basement membranes. MMPs participate in several steps in tumor progression, such as invasion, metastasis and angiogenesis (Himelstein et al., 1995). MMP-9 has rapidly been associated with malignant tumor progression and metastasis by degradation of the matrix and to facilate tumor invasion into other tissues (MacDougall et al., 1995).

Enhanced expression of MMP-9 has been observed in cancers of breast, colon, lung, skin, ovary, prostate… (Egeblad et al., 2002). For many of cancers, the increase in MMP-9 activity was often correlated with malignancy status, increased invasiveness and increased metastasis. The current view is that gelatinases, and in particular MMP-9, are needed at multiple stages during the tumor progression such as growth of the primary tumor, intravasation of tumor cells and invasion of the metastatic cells in the secondary organ (Björklund et al., 2005). Angiogenesis is also a stage of tumor invasion in which MMP-9 plays a significant role by triggering the angiogenic switch during carcinogenesis via the release of vascular epidermal growth factor (Bergers et al., 2000).

2. NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN

2.1. The lipocalin family: a common tertiary structure for multiple and diverse functions

The lipocalin family is a large group of extracellular proteins that are widely distributed in the living organisms. They can be found in animals, as well as in plants, and even in bacterias. The name lipocalin is derived from the Greek words *kalyx* and *lipos*. This etymology reveals two fundamental aspects of this family: their three dimensional structure that is in the shape of a kalyx, and their ability to bind various lipophilic ligands, ability which will determine many properties.

The functions of those extracellular cup-shaped proteins are numerous and highly varied: pheromone activity, olfaction, regulation of immunological processes, coloration, cell regulation, developmental processes, reaction of organisms to various pathologies such as bacterial infections or tumoral processes and many more that remain unstudied until now (Grzyb et al., 2006).

2.2. NGAL general structure

Despite their low homology in the amino acid sequence, the lipocalins share a common tertiary structure and an overall folding pattern that is highly conserved and essential for their function. The region determining the specific properties of the lipocalin is the kalyx. It is formed by nine β–strands, a large alpha helix and a much smaller one, with only three turns, called the 3_{10}-helix. Eight of the nine β–strands are not only linked by β–hairpin connections, but are also connected by transversal hydrogen bonds and are organized in an antiparallel way forming a β–sheet. This β-sheet is closed back on itself with hydrogen bonds to form a β-barrel. The barrel has a closed end on one side and an open end which is the opening of the ligand-binding site and is partially closed by the Ω-loop, also known as the scaffold loop. The cavity inside the calyx is very rich in lipophilic amino acid residues, making the ligand-binding site very hydrophobic, and explaining the lipophilic nature of the ligands (Flower et al., 19995, 1996). NGAL is made up with 178 amino-acid residues (Kjeldsen et al., 1993) and respects the typical lipocalin secondary and tertiary structures (figures 2, 3, and 4).

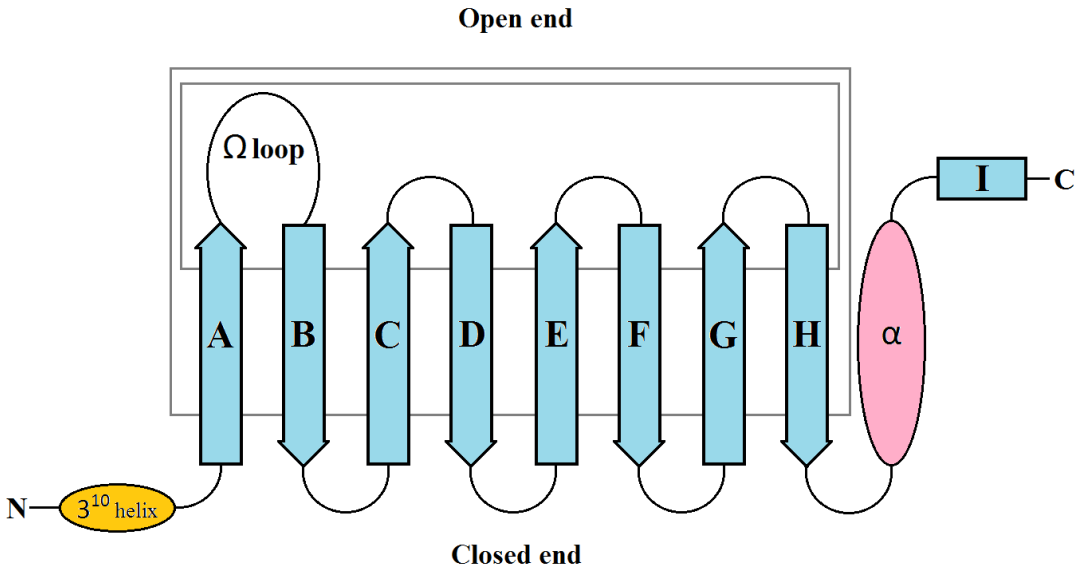


Figure 2: Unfolded NGAL: simplified view of the typical lipocalin fold, orthogonal to the axis of the kalyx

The antiparallel β-strands are labeled A-I and the α-helix is labeled α. The N-terminal 3_{10}-helix and the C-terminal are also marked. Hydrogen bonds are marked by the grey lines. Strand A interacts with strand H, stabilizing the domain (according to Flower et al., 1996).

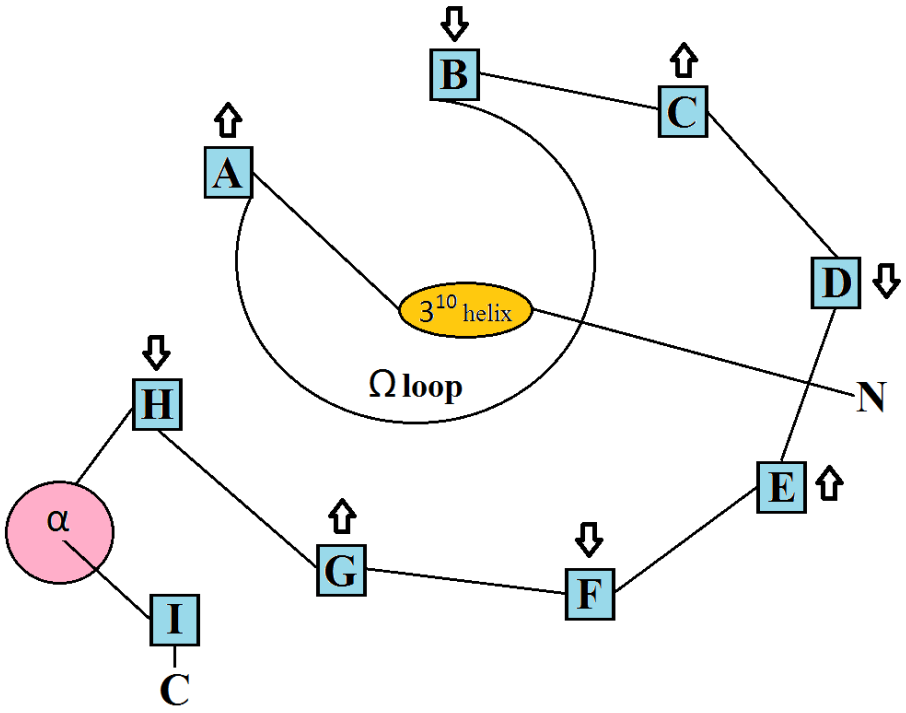
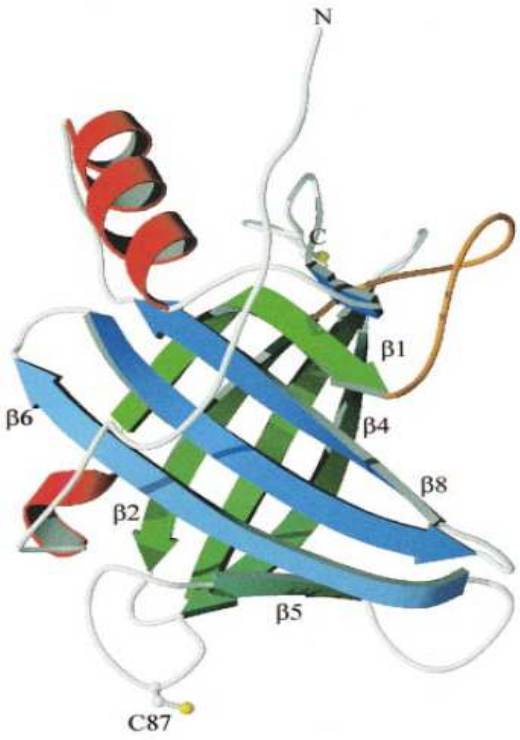


Figure 3: Simplified view of NGAL fold in the axis of the barrel (cross section)

β-strands marked with an arrow pointing up have a strand direction out of the plane of the paper and those with an arrow pointing down are directed into the plane of the paper (according to Flower et al., 1996).



<u>Figure 4</u>: NGAL's calculated structure according to Coles et al. (1999)

The view is orthogonal to the barrel axis.The β-strands of the two different β-sheets are shown in blue and green, the helix are labeled in red and the Ω-loop in orange. Note how C87 is projected into the solvent and is therefore highly accessible for protein-protein interactions, in particular with MMP-9.

- Interaction site with MMP-9

At the floor of the barrel, very close to Cys 87, three amino-acid residues (Asp 34, Glu 60, Asp 61) form a negatively charged patch. This site has been suggested to be the interaction site between NGAL and MMP-9, while the unpaired cysteine Cys87 forms the intermolecular disulphide bond between the two proteins (Coles et al, 1999).

2.3. Physiological functions of NGAL

First believed to be only transporters of small lipophilic molecules, lipocalins tend to prove that they fulfill many different biological functions, some potentially significant, such as immunomodulation (Kremer JM. et al, 1988), cell regulation (Nagashima M. et al, 2009), enzyme synthesis, olfaction and taste (Guiraudie G.et al, 2003), pheromones (Bacchini A. et al, 1992). NGAL, just like most of the other members of its family, shows a wide variety of these functions.

- Iron capture and bacteriostatic effect

Iron is a metal essential for microbial growth. However, when infecting a human person, they find themselves in a severely iron-poor environment as the estimated concentration of free iron in the human body is as low as 10^{-24} mol/L (Carrano et al, 1978). In response to this iron-poor environment, microbes, in particular bacteria, have developed siderophores (Greek meaning “iron carrier proteins”). Siderophores are relatively low molecular weight proteins produced by microorganisms whose affinity for iron, especially ferric iron (Fe^{3+}) is several times higher than the affinity of human endogenous iron-binding proteins. They can capture the iron from the surrounding environment and transport it back into the bacteria (Goetz et al., 2002).

NGAL has the ability to bind siderophores and to transport them inside the cells, preventing bacteria from acquiring the siderophore-bound iron they need. This mechanism is believed to underlie the main functions accredited to NGAL. Released by activated neutrophils, NGAL acts as a bacteriostatic agent and participates in the antibacterial iron-depletion strategy of the immune system (Goetz et al, 2002). The antibacterial function of NGAL was confirmed by Berger and colleagues in 2006: generated gene-targeted NGAL-deficient mice showed a significantly increased sensitivity to *E.coli* infection (Berger et al, 2006).

However, this lipocalin is also expressed in aseptic diseases, implying that it may serve additional functions.

- Management of intracellular iron concentration, specific cellular responses and role in cell apoptosis

After fixation with its cell-membrane receptor, NGAL is up-taken intracellularly and is stored in an endosome. If it contains an iron molecule, it will deliver it, generating an increase in intracellular iron concentration promoting regulation of iron-responsive genes like ferritin and transferrin receptors (Yang et, 2002). However, if NGAL is free of any iron, it will bind to intracellular iron before being exocytised (figure 5). In this case, NGAL has an action opposite to the one it has if it contains iron, the regulation of iron-responsive genes is in the

opposite direction, and if the intracellular iron concentration is massively decreased, cell apoptosis can ensue.

Ectopic expression of NGAL receptors on HeLa cells confered them the ability to undergo either iron uptake or apoptosis, depending on whether the ligand of NGAL was iron-loaded or iron-free. NGAL:siderophore:iron complex significantly increased the intracellular iron amount whereas NGAL:siderophore free of any iron molecule, generated a decrease in the intracellular iron concentration, and resulted in apoptosis (Devireddy et al, 2005).

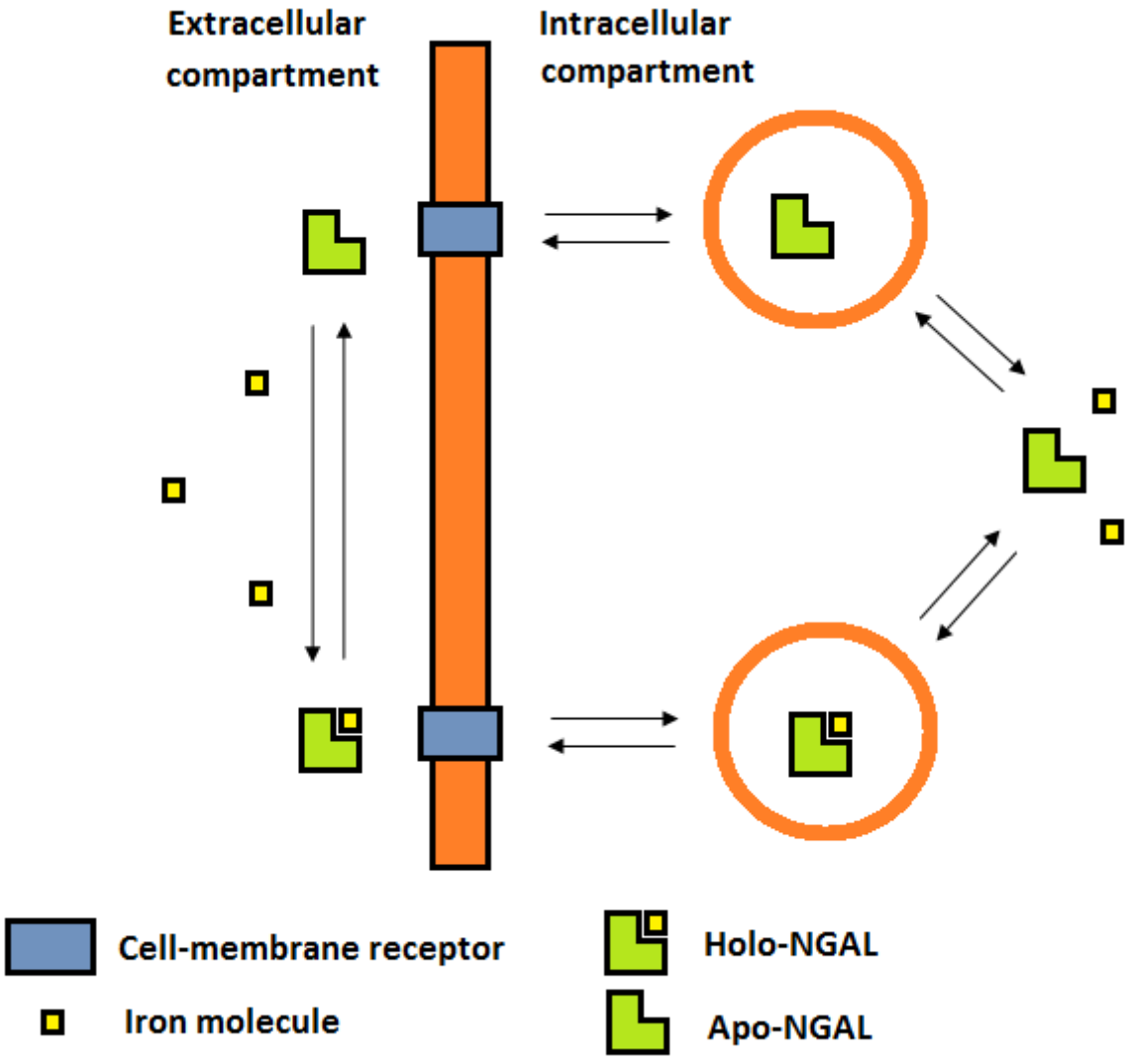


Figure 5: Schematic depiction of NGAL cellular cycle

The binding of NGAL with its receptor is followed by the internalization of NGAL and its ligand. Holo-NGAL can then release its iron molecule, inducing an increase in the intracellular ferric concentration and also regulating iron-dependent metabolic pathways.
Apo-NGAL can also interact with the same receptors and be internalized. Once inside the cell, it can bind to the iron present inside the cytoplasm and be externalized, in the exact opposite way as previously described with holo-NGAL. The intracellular ferric concentration will therefore decrease (according to Bolignano et al, 2010).

- NGAL is a pro-inflammatory molecule and a neutrophil chemoattractant

NGAL expression is strongly up-regulated in several inflammatory diseases: psoriasis (Mallbris et al, 2002), periodontitis, myocarditis (Yndestad et al, 2009), ischemia reperfusion injury (Lee et al, 2011) and ulcerative colitis (Galamb et al, 2008).
While studying the mediation function of NGAL in immune responses to bacterial infection, it was observed that NGAL's expression was massively increased in the serum, the liver and the spleen of the tested mice, six hours after an intraperitoneal injection of *E.coli* (Flo et al, 2004).

NGAL is suspected to be a neutrophil chemoattractant as the number of neutrophils recruited to infiltrate heart in a mice model of heart-transplant was shown to be significantly higher in wild type mice than in NGAL knocked-out mice (Aigner et al, 2007). However, NGAL precise functions and factors of regulation in the inflammatory processes remain unclear.

- NGAL as a protective factor against oxidative stress

NGAL seems to play an important role in the antioxidant machinery of the cells. The first sign of this aptitude came in 2008, when Roudkenar et al. revealed that NGAL acted as a protective factor against hydrogen peroxide (H_20_2) toxicity. Using Chinese hamster ovary (CHO) cells, it was shown that the cell proliferation was higher in the CHO cells expressing NGAL compared to the control cells when H_20_2 was added in the culture. Conversely, H_20_2 was more toxic to the cells for which NGAL's RNA had been silenced (Roudkenar et al, 2008).

Experiments involving human embryonic kidney cells demonstrated that the NGAL expression had a significant modulation effect on the expression of heme oxygenase (HO1) and of superoxide dismutases (SOD 1 and SOD 2), which are both strong antioxidant enzymes. Overexpression of NGAL resulted in an upregulation of mRNA and protein translation of the enzymes, while silenced cells showed a diminution of the HO1 synthesis. Finally, the cells that were overexpressing NGAL were proven to be significantly more resistant *in vitro* to the strong oxidative stress due to the addition of hydrogen peroxide (Bahmani et al, 2010).

- Role in development

NGAL can mediate processes of growth, development and differentiation of several human tissues in the embryonic stages. Not only it was demonstrated that NGAL was a key participant in mesenchymal-epithelial transformation, due to its aptitude to increase cellular iron stores in the developing kidney (Yang et al, 2002) but, it was also shown that NGAL had more differentiation-inducing functions. In cultured collecting duct cells, hepatocyte growth factors stimulate epithelial cells to produce NGAL and when this expression was blocked by silencing RNAs, it induced cystic structures rather than correctly assembled tubules. These experiments demonstrated a regulatory role of NGAL in epithelial morphogenesis by mediating the organization of the cells (Gwira et al, 2005).

2.4. Function of NGAL in solid tumors

The role of NGAL varies depending on the type of cancer. In some organs, the protein will tend to have a positive role in the protection of cancer cells, therefore allowing the tumor to grow and metastase. Examples of such tumors are thyroid carcinoma (Iannetti et al., 2008), endometrial carcinoma (Miyamoto et al. 2011) or breast cancer. However in other types of cancers, the protein can sometimes have a completely opposite role and help the body to defend against the tumor by reducing its growth, decreasing its invasiveness and tendency to metastase or by inhibiting angiogenesis. It was shown to be the case in colon (Lee et al., 2006) and pancreatic cancers (Tong et al., 2008).

2.5. Effect of NGAL on MMP-9

Yan and co-workers showed by immunoprecipitation that NGAL could bind MMP-9 to form a complex. The formation of the complex was confirmed by *in vitro* reconstitution experiments by mixing the two proteins. Most interestingly, degradation of MMP-9 was significantly inhibited in the presence of NGAL and resulted in the preservation of its enzyme activity. This protective effect of NGAL was then confirmed in a cell-culture system in which forced expression of NGAL in human breast carcinoma cells resulted in an increase of MMP-9 enzymatic activity independent of changes in MMP-9 gene translation. It was thereby proven that NGAL could modulate MMP-9 activity by protecting it from degradation (Yan et al., 2001). It was then suggested that the involvement of NGAL in tumor progression may be in part due to its protective effect on the matrix degrading enzyme.

3. USE OF MATRIX METALLOPROTEINASE 9, NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN AS BIOMARKERS IN MAMMARY GLAND TUMORS

3.1. MMP-9 and NGAL in human breast cancer

The expressions of MMP-9 and NGAL in breast cancer have been thoroughly studied in human medicine these past years as the role of the two proteins in the tumoral progression soon became undeniable.

- NGAL can protect MMP-9 from degradation and increase tumoral growth rate

After it was shown that the binding of NGAL to MMP-9 protected the extracellular matrix remodeling enzyme from degradation, Fernàndez and coworkers hypothesized that the addition of NGAL to MMP-9 expressing breast cancer cells might result in a more aggressive type of tumor after implantation in immunodeficient mice. Human breast cancer cells were transfected with human NGAL expression constructs and were implanted in mice in order to monitor the growth and aggressiveness of the tumor. Provoked NGAL expression increased significantly the growth rate of tumors as well as the expression of MMP-9, the angiogenesis and the tumor-cells proliferation. This study also showed by gelatin zymography that the NGAL/MMP-9 complex was detected in the urine of 86.36% of the tumor-implanted mice and none in the healthy controls (Fernàndez et al., 2005). This last result confirmed the findings of Shen et al who demonstrated on a sample of 97 breast cancer patients that MMP-9 and NGAL expressions were valuable early prognostic factors in breast cancer (Shen et al., 2003).

- Serum concentrations of NGAL and MMP-9 can be markers of breast cancer status

Provatopoulou and co-workers focused their work on the evaluation of serum concentrations of MMP-9, NGAL, and the complex form in 113 women with breast abnormalities undergoing breast biopsy and 30 healthy women to serve as negative controls. They showed by immunoenzymatic assays that both the expressions of NGAL and MMP-9 as well as the NGAL/MMP-9 complex were significantly increased in patients suffering from invasive carcinoma compared to the healthy patients. The serum concentration level of MMP-9 and NGAL were also significantly correlated with the disease severity score, but not of the complex form. Their work thereby suggested that MMP-9 and NGAL could be used as biomarkers of breast cancer status and that the serum measurement of the two proteins by immunoenzymatic assays could be a non-invasive method to monitor the progression of breast cancer (Provatopoulou et al., 2009). These results were emphasized in 2011 when Sung and co-workers demonstrated that elevated serum concentrations of both MMP-9 and NGAL were associated with reduced disease-free survival in 303 breast cancer patients (Sung et al., 2011).

- MMP-9 is strongly expressed by the tumor cells

The immunohistochemical staining with an anti-MMP-9 antibody of breast tumor cells also showed significant results. It was demonstrated that for 84.8% of 138 patients suffering from breast carcinoma, the tumor cells were positive in semi-quantitative evaluation of the MMP-9 expression. Co-expression of MMP2/MMP-9 was a risk factor for patient survival but the relation between MMP-9 alone and survival rate was not significant. MMP-9 expression was also significantly correlated with other biomarkers frequently used such as estrogen and progesterone receptors (Ranogajec et al., 2012).

This work confirmed the first results obtained by Monteagudo and co-workers concerning MMP-9. They performed immunohistochemical assays on normal, benign and malignant breast tissues and demonstrated that epithelial cells exhibited strong immunoreactivity in 20 of 23 intraductal carcinoma cases. Invasive carcinomas were positive in 36 of 40 cases and metastatic cells in lymph nodes were stained in 10 or 12 cases. These results supported the role of the enzyme in tumor invasion and metastasis and suggested that tumor cells were the essential source of the enzyme in these processes (Monteagudo et al., 1990).

- NGAL expression in breast carcinoma tissue is a predictor of poor prognosis

Bauer and co-workers detected NGAL expression in 68 breast carcinomas in a cytoplasmic location. NGAL expression showed a significant correlation with poor histologic grade, presence of lymph node metastases and a high Ki-67 proliferation index. Its expression was also associated with decreased disease-free survival. NGAL detection may thereby provide information for risk assessment and may be useful to identify patients that may require a stronger adjuvant therapy (Bauer et al., 2008).

3.2. MMP-9 in canine mammary gland tumors

Because of the interest lifted by MMP-9 in human breast cancer articles, and because the human and canine mammary gland tissue share many similarities, few studies have been performed on canine MMP-9 to see if the results found in humans could be extrapolated to the canine mammary gland tumor.

Zymography assays first revealed that the intensity of MMP-9 activity in the mammary carcinoma tissue was significantly higher than in the benign tissues. The metalloproteinase activity in carcinomas was more than 10-fold higher that in the controls. The MMP-9 concentrations found in the sera of dogs suffering from mammary carcinoma were also higher than in healthy controls (Yokota et al., 2001). Similar results were obtained by Hirayama and co-workers (2002) and Kawai et al. (2006).

The same institute (Department of Veterinary Pathology at Rakuno Gakuen University) then studied the MMP-9 expression level in tumoral canine mammary tissue by immunohistochemistry with an anti human-MMP-9 antibody. It was shown that the expression of the metalloproteinase was very strong in epithelial cells of malignant tumors, and remained weak or moderate in benign tumors (Hirayama et al., 2002).

Very recently, Santos and co-workers immunohistochemically evaluated MMP-9 expression in benign and malignant mammary tumors (MMT) in the aim to relate the expression to prognostic factors and patient outcome. By studying the staining of 118 different mammary tumor tissues, they showed that the MMP-9 expression was correlated with malignancy status, high proliferation, invasive growth, high histological grade and metastatic capacity. The assays were here again performed with an anti human-MMP-9 antibody (Santos et al., 2012). Their study was published few weeks after the beginning of this study.

3.3. NGAL in veterinary medicine

To our knowledge, no article concerning canine NGAL studies has yet been published and this is the first study on canine NGAL and its relationship with mammary gland tumors.

EXPERIMENTAL WORK

EVALUATION OF THE EXPRESSION OF MMP-9 AND NGAL IN SERUM, URINE AND TUMORAL TISSUES OF FEMALE DOGS SUFFERING FROM MAMMARY GLAND TUMORS

1. MATERIAL AND METHOD

1.1. Production of anti-canine-MMP-9 antibody

Foreword

The canine MMP-9 antibody production was a major part of this study. The report of its production requires the insertion of several intermediate results, essential to link one step to the next, thereby resulting in a substantial material and method section.

1.1.1. RNA extraction and reverse transcription

- Total RNA extraction

Tissue sample (0.2 g) was extracted from canine testicule with a surgical blade and the cells were lysed using liquid nitrogen, a mortar and a pestle. Total cell RNA was extracted using Qiagen®'s RNeasy® kit. The kit relies on using a spin-down column with a positively charged silica-based membrane that will retain the negatively charged RNA while letting cell debris, proteins and other unwanted components flow through. Firstly the sample was lysed and homogenized in a highly denaturing buffer (RLT buffer) which inactivates RNase enzymes. A high-salt buffer system and the presence of ethanol to enrich binding conditions then allowed total RNA longer than 200 bases to bind to the membrane. Contaminants were washed away during the centrifugation and the total RNA (50 µl) was then eluted with Elution Buffer (Protocol 1 in Appendix 1).

- Reverse transcription (RT)

The total RNA was transcribed into cDNA using biological reverse transcription. (Invitrogen™'s Superscript™ III Supermix kit for qRT-PCR). The kit contains RT Reaction mix (contains the buffer, poly dT primers, random primers and 0.1M DTT) and RT Enzyme mix (contains reverse transcriptase and RNase inhibitors). The reverse transcription is performed by mixing the RT Reaction mix and Enzyme mix with the RNA sample and DEPC treated water and running the PCR machine for 1 cycle (Protocol 2 in Appendix 1).

1.1.2. Production of a cDNA insert

- MMP-9 Hydrophobicity analysis and choice of the primers

The aim was to produce 3 different MMP-9 antigens: one which would be the full length protein and two others which would only be a portion of the molecule, thereby inducing the formation of more specific antibodies. As the purpose of the antibodies produced from those antigens was to detect native form protein, the aim was to reproduce very hydrophilic portions of the protein, as such regions have high chances to be protruding in the environment rather than hydrophobic regions which have high chances to be inaccessible in the native protein.

The expected hydrophobicity of canine MMP-9 was analysed using Kyte J and Doolittle R's method (Kyte et al., 1982) and the proximal part as well as the distal part of MMP-9 showed a lower hydrophobicity than the rest of the molecule. It was decided to also produce two partial antigens: one would be the first quarter of the protein and the second one, the last quarter, each of them being around 170-200 amino acids (figure 6).

- Polymerase Chain Reaction (PCR)

Four different primers were thus ordered:

MMP-9-1F-35: 5'-AACATATGCAGCCCCTGGTCCTGGTGTTC-3' (29 nucleotides)

MMP-9-1R-544: 5'-AACTCGAGAACACCAAACTGAATGATGATG-3' (30 nucleotides)

MMP-9-1F-1580: 5'-AACATATGGCCATCGCGGAGATCAGGAACTAC-3' (32 nucleotides)

MMP-9-1R-2131: 5'-AACTCGAGGCACTGCAAAATGTCAAAG-3' (27 nucleotides)

With the 4 primers ordered, 3 different PCR reactions were attempted (Appendix 2):

- MMP-9-1F-35 and MMP-9-1R-544 (expected length: 500bp): obtained fragment should be the proximal part of MMP-9, which will be referred to as MMP-9-Prox.
- MMP-9-1F-35 and MMP-9-1R-2131 (expected length: 2100bp): obtained fragment should be the full MMP-9 molecule, which will be referred to as MMP-9-Full
- MMP-9-1F-1580 and MMP-9-1R-2131 (expected length: 550bp): obtained fragment is the distal part of MMP-9, which will be referred to as MMP-9-Dist (figure 7).

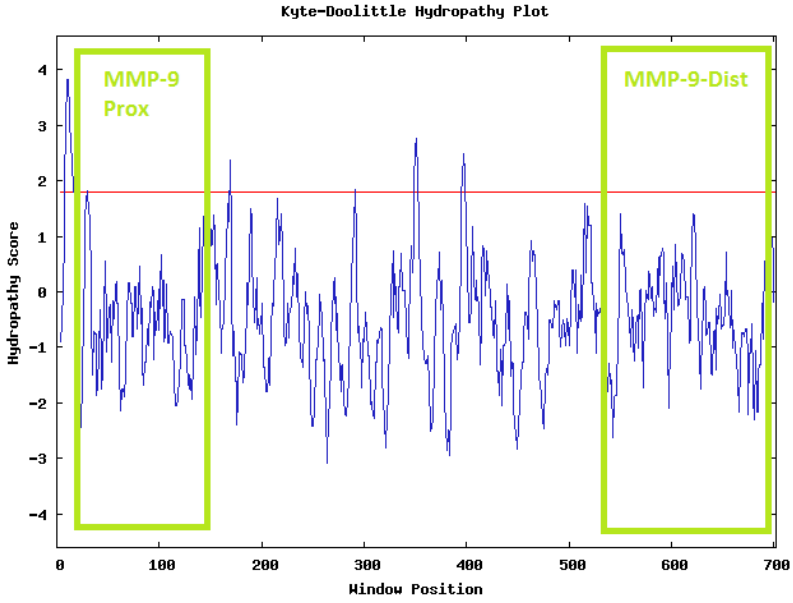


Figure 6: Hydrophobocity analysis of canine MMP-9

The regions showing the lowest hydrophobicity scores have more chances to be detected on the native protein. MMP-9-Prox and MMP-9-Dist are labeled and it can be noticed that no sequence in these two regions reaches a high hydrophobicity score.

The template DNA was mixed with the forward primer, the reverse primer, nucleotides, DNA polymerase and 35 PCR cycles were performed (Protocol 3 in Appendix 1). Agarose gel electrophoresis (Protocol 4 in Appendix 1) was used to check the PCR products (figure 7).

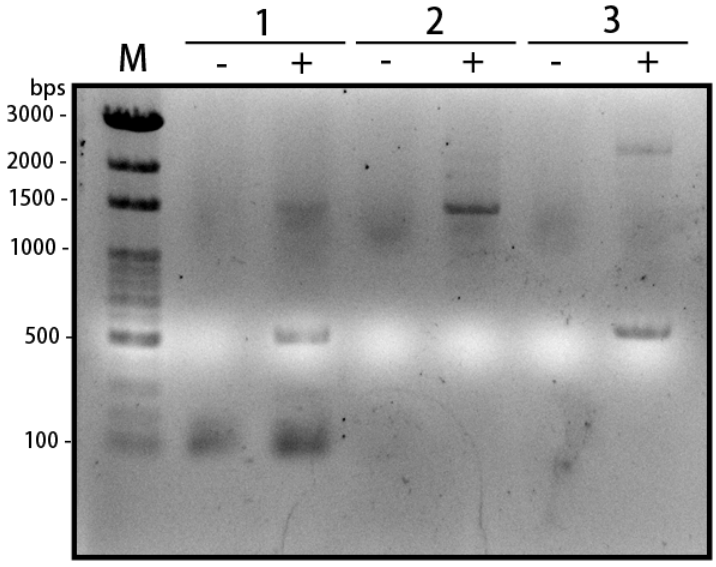


Figure 7: Agarose gel electrophoresis to verify the size of the PCR products

The three reactions MMP-9-Prox, MMP-9-Full and MMP-9-Dist are labeled 1, 2 and 3 respectively. The negative controls are ddH2O only.

The fragments obtained from reactions 1 (MMP-9-Prox) and 2 (MMP-9-Dist) were compatible with the expected size. However, the amplification of the region coding for the full length MMP-9 seemed to have failed as the obtained fragment was 1500bp, compared to the 2100bp expected. This reaction was repeated several times but it always failed to give us a fragment of the right size. The project to create a full length antigen was stopped here.

1.1.3. Introduction to Novagen's pET-24a(+) vector

The vector used in this project was Novagen's pET-24a(+) (Appendix 3). This plasmid has a kanamycin resistant gene that was used in later steps as a selection marker and also offered the possibility of adding a histidine-tag at one end of the antigen. The histidine-tag is an alignment of 6 histidine residues that can be added at the C- or the N-terminus of the protein. It is poorly immunogenic, and at pH 8.0, it is small and uncharged, thus not affecting the antigenic properties of the protein. This tag gives the protein a very high affinity for Ni-NTA (nickel charged iminodiacetic acid) therefore offering the possibility to use this specific binding as a purification method once the protein has been expressed. Another interesting advantage is that it makes the protein easily detectable by anti-histidine-tag antibodies.

In this study a pET-24(a) plasmid that already contained a 1700bp insert was used.

1.1.4. Digestion of the vector, digestion of the insert and ligation

- Digestion of pET-24a vector

In order to ligate the insert inside the vector, both of them needed to be digested with the same restriction enzymes, namely *Nde I* and *Xho I* (figure 8). These two enzymes were chosen because both have a unique cutting site in the vector's sequence, none of them has a cutting site in the sequence of the MMP-9 insert and it put the His-tag at the C-terminal of the protein.

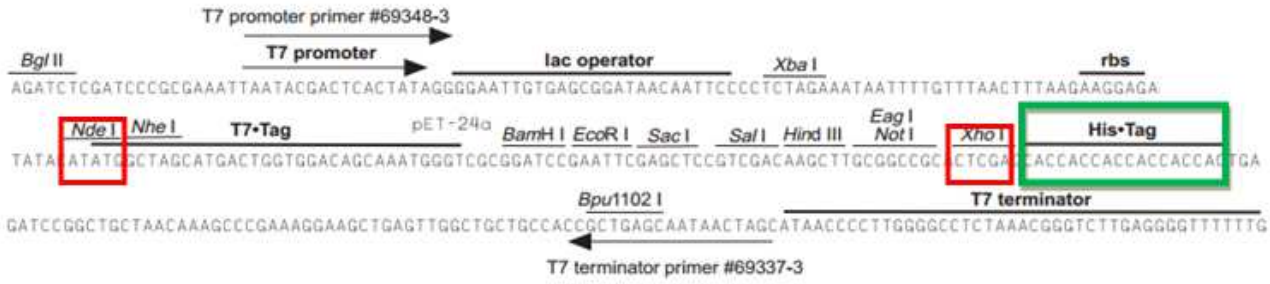
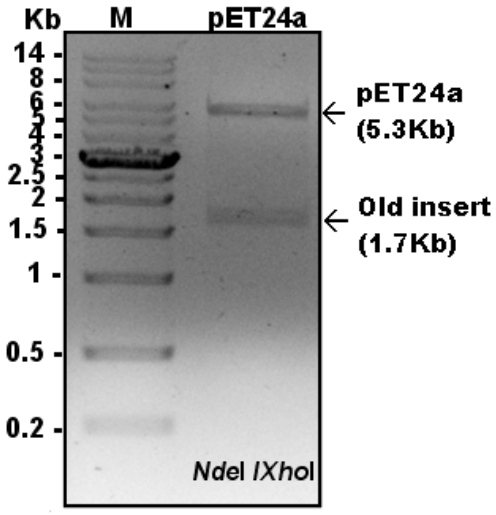


Figure 8: Map of pET-24a(+) expression region

The enzyme restriction sites used (*Nde I* and *Xho I*) are shown in red. The histine-tag, which will be used in the purification of the protein is marked in green.

The choice of the enzymes was made before ordering the primers. It allowed to input a cutting site inside the primer, thereby giving the insert a *Nde I* and a *Xho I* cutting site at both extremities.

To perform the digestion, the plasmid DNA was added in an Eppendorf tube containing ddH2O (double distilled water), BSA, *Nde I* and *Xho I* and incubated at 37°C during 2 hours (Protocol 5 in Appendix 1). The solution (4 µl) was then checked on a 1% agarose gel electrophoresis. Because the plasmid already contained a 1700bp insert, two marks were expected: the first one around 5000bp, corresponding to the digested plasmid, and the second one at 1700bp, which matched with the excised insert. The digestion was judged successful as both components could be detected (figure 9).



<u>Figure 9:</u> Products of pET-24a digestion by *NdeI* and *XhoI* on 1% agarose gel electrophoresis

The 5000bp mark was then cut from the gel and eluted by using Geneaid™'s Gel DNA Fragments Extraction Kit. This ensures us to farm only the digested plasmid and to get rid of the old 1500bp insert (Protocol 6 in Appendix 1).

- <u>Digestion of the cDNA insert</u>

The cDNA inserts of MMP-9-Prox and MMP-9-Dist were digested with the same enzymes, using the same protocol as the one used for the vector (Protocol 5 in Appendix 1).

- <u>Ligation</u>

The ligation is the operation in which the DNA of the protein is inserted into the plasmid vector (figure 10). The proportions of plasmid and insert to use in the reaction were determined by the signal intensity of those two components on a 1% agarose gel electrophoresis. The aim was to have 2-3 times more insert than plasmid, in order to increase

the competitivity of the desired reaction and to minimize the risk of obtaining self-ligation (i.e. plasmid closing back on itself without integrating the DNA insert). The ligation is performed by mixing the DNA insert with the plasmid and DNA ligase enzyme and leaving the mixture at 4°C overnight (Protocol 7 in Appendix 1).
The ligation could also be carried out at 16°C or even 37°C but the low temperature, though making the reaction slower, increases its specificity and minimizes unwanted reactions such as self-ligation, therefore increasing the final yield of successfully ligated plasmids.

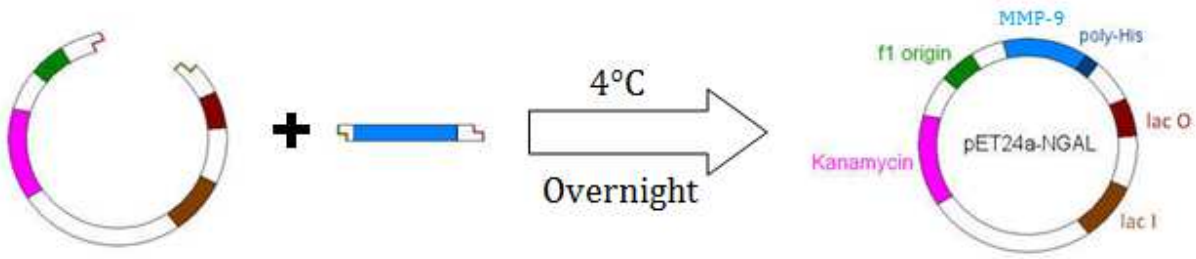


Figure 10: Schematic view of ligation between the cDNA insert and the pET-24a vector

1.1.5. Transformation

The pET-24a-MMP-9 plasmid was then inserted inside competent *E.coli* cells. The bacteria used in this study were One Shot® Top10 competent *E.coli* from Invitrogen®. These bacteria have very high transformation efficiency and are ideal for high-efficiency plasmid cloning as they allow stable replication of plasmids in a high-copy number. It is important to mention that these bacteria contain no plasmid, which means that the only one that will be present after the transformation were pet24a-MMP-9. However, this also means that the Top10 *E.coli* do not have the necessary cellular equipment to express the protein. They were used to clone the plasmid prior to transforming them in another type of bacteria (BL21) that contains other plasmids as well as all the cellular machinery needed to produce the antigen.

- Preparation of the competent cells

The first step consisted in preparing the competent cells by refreshing an overnight culture and incubating it until it has reached the logarithmic phase of its growth curve.
This moment was determined by spectrophotometry at 600nm. By using a clean LB-broth sample as standard, the log phase starts when the sample's optical (OD) was between 0.3 and 1. When the OD reached 0.4, the growth was stopped. The bacteria pellets were centrifuged and cleaned with 0.1M $CaCl_2$ washes. $CaCl_2$ was used to positively charge the bacteria, thus increasing its attraction to the negatively charged DNA plasmid, and increasing the chances of successful transformation. Glycerol was also added to enable the stocking at -80°C (Protocol 8 in Appendix 1).

- Transformation

The aim of the transformation step was to mix the negatively charged plasmid DNA with the positively charged competent cells and to use a heat shock to make the cells permeable enough to acquire the plasmid. At first the two different components were mixed and left on ice, in order for the plasmid and the cells to get closer to one another as they are attracted by their opposite charges. The mixture was then quickly bathed at 42°C. The sudden increase of temperature was used to enlarge the pores of cell membrane and increases the cell permeability, therefore allowing the pET-24a-MMP-9 plasmid to enter the cell during this short period of time. The sample was then quickly thawed on ice, in order to close the bacteria's pores and to trap the plasmid inside the cell (Protocol 9 in Appendix 1).

- Colony selection

LB broth free of any antibiotic was then added into the tube and the sample was incubated for a short period of time in order to allow the bacteria to start their growth. A small volume of the bacteria culture was streaked onto a LB agar plate containing kanamycin. Top10 *E.coli* are naturally sensible to kanamycin and only those that have captured the plasmid containing a kanamycin resistance gene should be able to grow. The culture plate is incubated at 37°C for 18 hours and the resistant colonies were selected (Protocol 10 in Appendix 1).

The kanamycin plate allowed to keep only bacteria that have gone through a successful transformation. This means that any bacteria which managed to develop on the plate must have received a pET-24a(+) plasmid, but it was of no guarantee that the plasmid it had gotten contained the MMP-9 DNA insert, as it could be a self-ligated plasmid. After this colony selection a colony screening has to be performed.

1.1.6. Colony screening

Three different methods (PCR screening, analysis of enzyme digestion pattern, and sequencing) were used in order to verify that the selected colonies had a pET-24a(+) plasmid containing the MMP-9 DNA sequence insert. Those methods were

- PCR screening

For both MMP-9-Prox and MMP-9-Dist, 20 colonies were selected from the kanamycin plates and were amplified using the corresponding primers.

Single colonies were picked from the LB plate and resuspended in 30 µl of ddH_2O. Sample fractions (2 µl) were tested by PCR and the remaining fraction (28 µl) would be used for bacterial inoculation if the colony was proven to be positive. The screening by PCR was performed by mixing the resuspended bacteria with the forward and reverse primers used in Protocol 3 as well as with DNA polymerase enzyme and nucleotides.

The sizes of the PCR products were then verified with a 1% agarose gel electrophoresis (figure 11). The colonies showing the strongest signal on the agarose gel were selected for the next screening test.

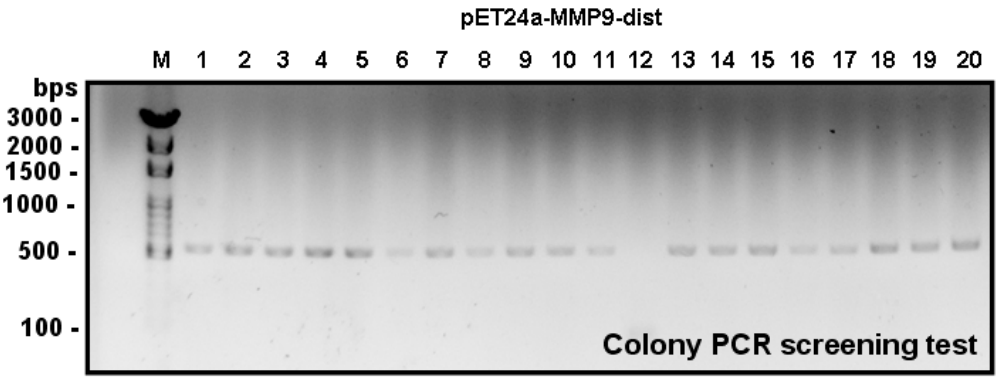


Figure 11: PCR products obtained for 20 colonies after transformation with pET-24a(+) carrying MMP-9-Dist insert (1% agarose gel electrophoresis)

Most of the colonies selected showed a successful transformation as the PCR product size was around 500bp, matching with the size of the MMP-9-Dist insert**.**

- Enzyme digestion pattern

After the overnight incubation of the LB agar + kanamycin plate, one of the isolated colonies was selected and was grown in 3 ml of LB broth containing kanamycin. The bacteria developed and cloned the plasmid they had received. The plasmid clones were then extracted using a Geneaid® *High Speed Plasmid Mini Kit* protocol. The principle of the kit was to use alkaline lysis and RNase treatment to obtain clear cell lysate with minimal genomic DNA and RNA contaminants. The plasmid DNA in the lysate binds to the glass fiber matrix of the *PD Column* that is provided in the kit. Contaminants could then be removed with a wash buffer containing ethanol and the purified plasmid DNA eluted using a low salt elution buffer (Protocol 11 in Appendix 1).

After elution, the plasmid DNA was digested using *Nde I* and *Xho I* as restriction enzymes. Two fragments were expected in 1% agarose gel electrophoresis: the first at 5000bp, corresponding to the digested pET-24a(+) plasmid, free of any insert, and the second one around 500bp matching with the size of the inserted MMP-9 DNA fragment (figure 12).

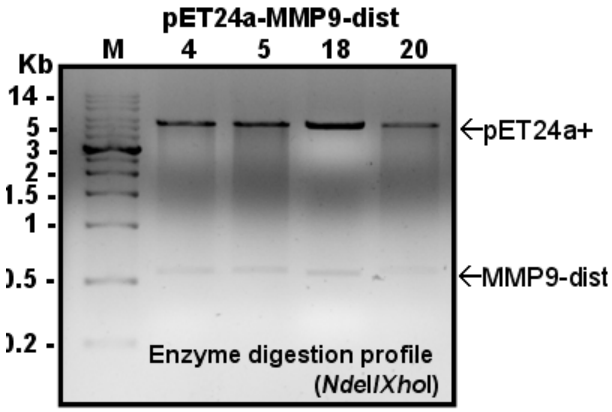


Figure 12: Enzyme digestion profile of the plasmids extracted from colonies after transformation with pET-24a vector carrying MMP-9-Dist insert

Only two signals can be noticed for the four samples, one belonging to the vector, the second one to the 550bp MMP-9-Dist insert (1% agarose gel electrophoresis).

- Sequencing

Finally, a LB plate containing isolated colonies of the clones was sent to Mission Biotech laboratory in Taipei to sequence the plasmid. For both MMP-9-Prox and MMP-9-Dist, the sequencing result was identical to the selected portion of MMP-9.

1.1.7. Transfer of the plasmid to BL21 *E.coli*

The Top10 *E.coli* allowed to clone in a large-copy number the plasmid that contained the DNA insert coding for the antigen, but these bacteria, free of any natural plasmid, did not have the necessary cellular equipment to produce the antigen. Therefore, the plasmid was transferred to a new bacterial host (Biomol®'s BL21 which is one of the most widely used bacterial gene expression host) able to translate and express the sequence it contains. BL21 is able to grow in minimal medium and is deficient in key proteases such as *lon* and *ompT*. *Ion* is an intracellular protease whose purpose is to lyse proteins considered abnormal by the host; it would thus degrade the protein before the cells are even lysed. *ompT* is an extracellular protease that would degrade the proteins after the cells are lysed. The deficiency in the two main proteases and the T7 promoter expression system they carry enable BL21 to produce proteins in a large-copy number.

The transformation was performed with the same protocol as the one used for the Top10® bacteria. The BL21 were mixed with the plasmid clones eluted with Geneaid®'s *High Speed Plasmid Mini Kit* protocol, and a heat shock enabled the plasmids to penetrate inside their new host (Protocol 9 in Appendix 1).

The bacteria that had received the plasmid were then selected by streaking the transformed BL21 on an LB plate containing kanamycin (50 µg/ml).

1.1.8. Large scale expression and protein extraction

- Antigen expression

A single colony was picked from the LB plate and incubated in a 10 ml kanamycin enriched LB broth culture at 37°C overnight. The culture was then refreshed at a 1/20 ratio into a new medium and incubated at 37°C for about 2 hours, until the optical density reached 0.6 at 595nm. A fraction (1 ml) was sampled from the culture, and was stored at -20°C as the "no induction" control. The rest of the culture was induced with 0.8 mM IPTG.

Though MMP-9-Prox could be produced in small scale expression tests (Protocol 12 in Appendix 1), it seemed impossible to produce it in large scale. Many different conditions have been attempted, such as different IPTG concentration (0.2-2 mM), expression temperature (25-37°C), expression time (2-24 hours), but all of them resulted in a production extremely low if not completely absent. After several repeats, the project to create recombinant MMP-9-Prox was aborted.

For MMP-9-Dist, several different expression conditions have been experimented and the optimal expression protocol was an incubation at 25°C for 24 hours (Protocol 13 in Appendix 1).

- Antigen extraction

The aim of the next steps was to lyse the bacteria in order to free the protein. For that, freeze (-80°C) and thaw (37°C) cycles were used in order to inflict to the bacteria an important heat shock. To make sure the bacteria were lysed, lysozyme was added and the sample was put in a 37°C waterbath. Sonication with ultrasounds was then performed. The sample was centrifuged and the supernatant and pellet were separated.

The native protein is soluble; therefore it was dissolved in the supernatant. However, the denatured form, which in this case represented the majority of the protein, was still trapped in the pellet. To make it soluble, the pellet was resuspended with a new buffer, containing a high concentration of urea (8M) and salt (1M) and repeated the steps of sonication and centrifugation (Protocol 14 in Appendix 1).
The fact that the protein should have six histidine residues (his-tag) at the C terminal end was used to purify the antigen.

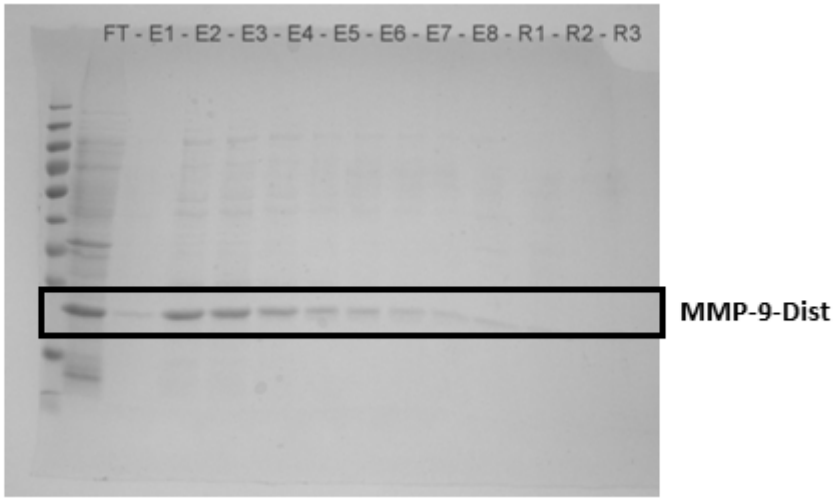
Some amino-acids, such as histidine, can form complexes with metal ions, in particular the nickel ion Ni^{2+}. If this ion is immobilized on a chromatographic medium by chelating to a suitable ligand, the amino-acids can bind to the medium and the proteins showing great proportions of them can be retained while the other proteins can be washed off. This is the principle of immobilized metal ion affinity chromatography (IMAC).
GE Healthcare's Chelating Sepharose™ contains iminodiacetic acid groups coupled to sepharose. After being charged by nickel, it can bind very efficiently to sequences alignement

that have multiple histidine residues next to each other. Therefore its binding to the MMP-9-Dist should be much more important than its binding to the bacteria endogenous proteins.
This binding can be neutralized by adding an imidazole enriched buffer. The resistance of the binding depends on the affinity between the beads and the proteins, and the stronger this affinity is, the higher the required imidazole concentration will be.
Therefore the first washes were done with 20 mM and 50 mM imidazole solution, in order to unbind all the proteins that are not highly bound to the sepharose. The proteins with a histidine-tag were highly bound and should not be affected by such low imidazole concentrations. The protein content of the flow-through of the washes was tested in protein dye. When this test revealed that the flow-through had a very low protein concentration, all elements that were unspecifically bound to the sepharose had been washed away and the elution process was started.

The only difference between the washing buffer and the elution buffer was the concentration in imidazole. This time, instead of being 20 mM and 50 mM, it was 100 mM for the first elutions and 400 mM for the last one. Here again, the protein content was checked with protein dye and when it was very low, the protein was considered to be eluted. The aim of the last steps of the protocol was to wash the column of sepharose with a regenerating buffer that contains EDTA (Protocol 15 in Appendix 1).

Finally, a SDS-PAGE (Protocol 16 in Appendix 1) was run in order to check the distribution of the protein in the different samples (figure 13).

MMP-9-Dist was the major protein in the eluted samples but a lot of contaminants were also visible. Further techniques were thereby applied to increase the purity of the antigen.



<u>Figure 13</u>: Verification of the histidine-tagged protein purification by SDS-PAGE after an expression done at 25°C for 24 hours

The Flow-through that did not bind is labeled *FT*, the different elutions are labeled *E1-E8* and the regeneration flow-throughs are marked *R1-R3*.

1.1.9. Protein purification

- Extraction of the protein from PVDF membrane

The proteins of the different elution samples were then separated by SDS-PAGE and transferred on a PVDF membrane. The membrane was then stained with Ponceau S and when the proteins became visible, MMP-9-Dist was located and cut. The piece of membrane was destained in ddH_2O for a few minutes and inserted in a new microcentrifuge tube containing an Elution Buffer. It was vortexed during 10 minutes and the membrane pieces were discarded. The elution efficiency was evaluated by checking with Ponceau S that the membrane pieces did not contain anymore protein (Protocol 17 in Appendix 1).

- Acetone precipitation

As the Elution Buffer used to elute the antigen from the membrane contained 2% SDS, it was not possible to use the sample for immunization before cleaning out all the SDS away. Regular dialysis was first thought to be the good option but it showed to be unsuccessful as the sample seemed to still contain a lot of SDS. Acetone precipitation was thereby chosen.

After adding a 4:1 volume ration of cold acetone the proteins precipitated. Afterwards centrifugation was performed to discard the supernatant that contained the Elution Buffer. The pellet was then resuspended in PBS. Therefore this technique also helped to concentrate the antigen by adding a small buffer volume to resuspend the pellet (Protocol 18 in Appendix 1).

1.1.10. Antigen concentration estimation

In order to estimate the protein concentration, a SDS-page was performed with the different samples and bovine serum albumin (BSA) standards of known concentrations (1000 µg/ml, 500 µg/ml, 250 µg/ml and 100 µg/ml). The gel was then stained and pictured and the signal intensity of the antigen was compared to the one of the standards using *Image J* computer software, and the concentrations were determined (Appendix 4).

The purity of the antigenic solution was also estimated by comparing the MMP-9-Dist concentration with the concentration of contaminants, eventually present in the sample. Any sample showing a purity rate lower than 50% was discarded or re-purified. If no other protein could be seen on the gel, the antigen was considered to be 99% pure.

1.1.11. Immunization and antibody retrieval

Prior to immunize, it was verified by Western Blot (Protocol 19 in Appendix 1) if the antigen was indeed the recombinant MMP-9-Dist. Using an anti-histidine antibody as the primary antibody, it was shown that the tested samples were very rich in a 22kDa protein that showed strongly positive for the histidine-tag (figure 14).

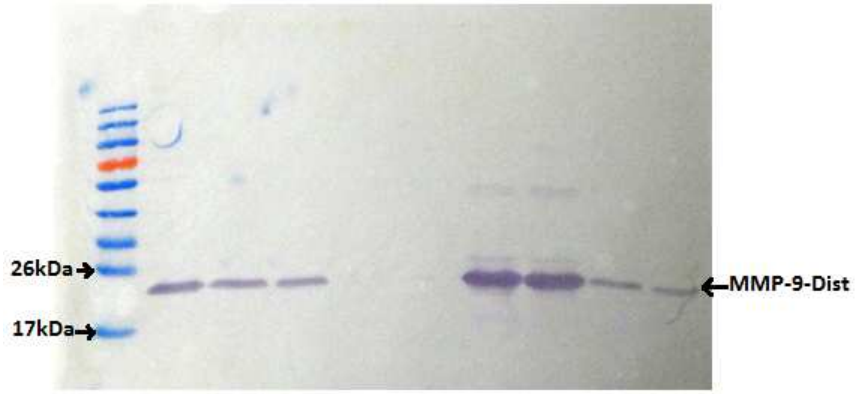


Figure 14: Western Blot result to verify the presence of the histidine-tag on the purified protein

The antigen was mixed with the adjuvant (Freund's adjuvant complete sigma) in a 1:1 volume ratio prior to being injected via intraperitoneous injection in three mice. One prime-injection and three boosting injections were performed for each mouse (Appendix 5).
Before each antigen injection, a blood sample was taken from the orbital sinus of each mouse. Blood was centrifuged at 6,000 g for 5 minutes; the serum was retrieved and stored at -20°C. The final serum was retrieved one week after the third boost and was used as a polyclonal antibody against canine MMP-9.

1.2. Production and purification of anti canine NGAL antibody

1.2.1. Recombinant NGAL production

The recombinant NGAL used to induce the production of antibodies was not produced during this study. This project only included the retrieval of the serum and the purification of the antibodies.
Mammary gland tissue was homogenized and total RNA was extracted. cDNA was synthesized using reverse transcription and the open reading frame of NGAL was amplified by PCR. The primers were designed according to GenBanl accession no. XM 548441.The gene was then cloned into a pET-32b vector and expressed in *E.coli*. strain BL21® (Invitrogen™) at 16°C for 24 hours after IPTG induction.

After centrifugation of the culture, the bacteria pellet was resuspended in lysis buffer containing a high concentration of urea and was subject to sonication. After a second centrifugation, the supernatant was mixed with the Nickel charged sepharose and incubated at 4°C for 2 hours. The sepharose was then washed several times and finally the bound protein was eluted and dialyzed against PBS at 4°C. The identity of the NGAL protein was confirmed by Western-Blot analysis using antibody against the histidine-tag and by mass spectrometry.

Three BALB/c mice aged eight weeks and three month old New Zealand white rabbits were immunized. Two boosters were given at two-week interval.

After the final bleeding, the sera were analyzed by Western-blotting. They were able to recognize the recombinant protein, whereas no signal was detected when the pre-immunization sera were tested. The sera were then tested by Western-Blot on clinical urine specimens. A protein of 25kDa, the expected size of endogenous canine NGAL, was detected in renal failure dogs but not in healthy dogs.

To use the antibodies for IHC, two purification methods were performed in order to enrich and concentrate the rabbit serum: ammonium sulfate precipitation and purification by protein G.

1.2.2. Antibody purification by ammonium sulfate precipitation

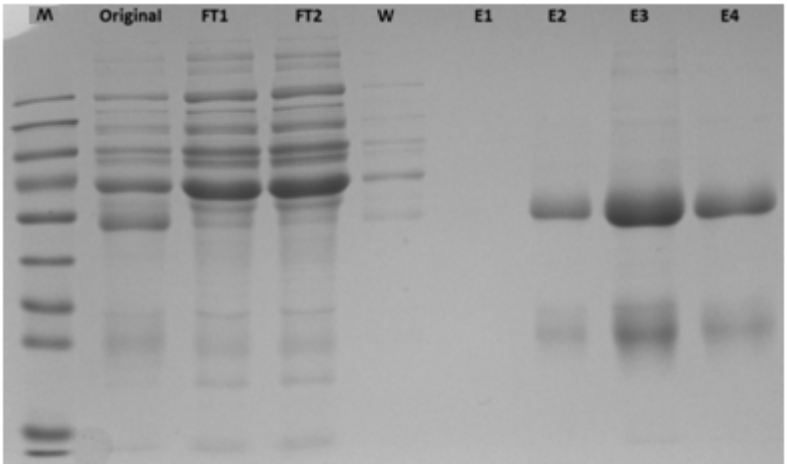
As the concentration of ammonium sulfate is increased in a sample, proteins become progressively less soluble until they precipitate. Antibodies precipitate at lower concentration of ammonium sulfate than most other proteins and serum macromolecules. At 40 to 50% ammonium sulfate saturation (100% saturation equaling to 4.32M), immunoglobulins precipitate while other serum proteins and components remain in solution (Protocol 20 in Appendix 1).

Ammonium sulfate precipitation provides sufficient purification for some applications but most often it is performed as a preliminary step prior another purification technique. The second technique used was column chromatography with Protein G.

1.2.3. Antibody purification by Protein G

This method relies on the fact that the protein G sepharose has a strong affinity for the Fc region of immunoglobulins. This property enables the G beads to trap the IgG and by running the rabbit serum on a column containing 2 ml of Protein G sepharose beads, it allowed to wash off the contaminants. The binding of the IgG was then cancelled by using a 100 mM glycine solution at pH3.0 and the antibodies were eluted (Protocol 21 in Appendix 1).

The eluted fractions were then checked on a SDS-PAGE (figure 15).



<u>Figure 15:</u> SDS-PAGE verification of the elutions retrieved after Protein G sepharose purification

The flow-throughs are labeled FT1 and FT2, the wash-through is labeled W and the elution fractions are labeled E1-E4 for fractions 1-4. Note that the heavy chain of the antibodies, at around 55kDa, was the major component of E2, E3 and E4 and most of the albumin had been discarded. The protein size ladder is labeled M.

1.3. Home-made NGAL Sandwich ELISA

1.3.1. Samples studied

The aim of the NGAL sandwich ELISA was to evaluate the expression of NGAL in the urine and the serum of female dogs suffering from malignant mammary gland tumors, and to compare it with the basal expression found in healthy dogs.

A total of 28 blood serum samples and 8 urine samples were obtained from female diseased dogs. All patients suffered from at least a simple carcinoma or a complex carcinoma or both and all their data was collected for statistical analysis.

1.3.2. Principle

Prior to start, serial dilutions of the recombinant canine NGAL were used as standards (from 5.6 µg/ml to 0.22 ng/ml).
To establish sandwich ELISA test, two antigen specific antibodies raised from different animal species, are required: two home-made canine NGAL antibodies, the first one originating from mice which will be the detecting antibody and the second one from rabbit which will serve as the capture antibody, were used in this study.

The principle was to make a coating with the rabbit antibody prior to apply the clinical samples. The capture mouse antibody was then applied and an enzyme-conjugated secondary antibody that binds specifically to the mouse antibody Fc region was then added. Finally, TMB as enzyme substrate of the enzyme conjugated to the secondary antibody was then used to obtain a color signal (figure 16).

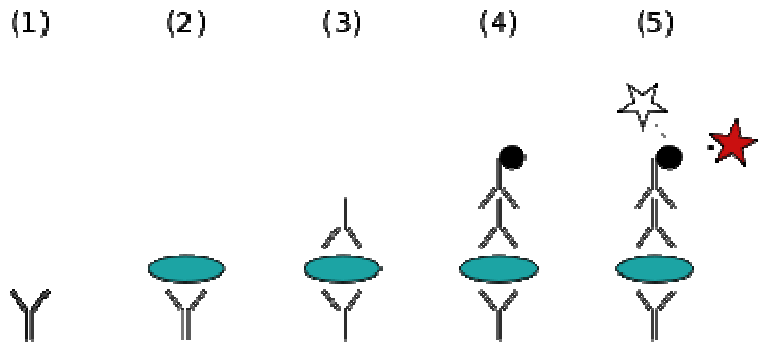


Figure 16: Schematic view of the NGAL sandwich ELISA principle

(1) Plate is coated with rabbit capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) mouse detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to mouse antibody; (5) substrate is added, and is converted by enzyme to detectable color form.

1.3.3. Optimized protocol

- Capture antibody coating

Rabbit anti-NGAL antibody diluted at a 1:800 rate in ELISA Coating Buffer was used as the capture antibody. All wells of the ELISA plate were filled with 100 µl and incubation was performed at 37°C for 1 hour. The wells were then washed 5 times with PBS.

- Blocking

150 µl of *ELISA Blocking Buffer* was added in each well and left 1 hour at room temperature before being washed off.

- Antigen addition

NGAL standards 1-8 and standard PBS were added in triplicates. For each clinical sample, 20 µl of the sample was diluted in 380 µl of PBS and three wells were filled with 100 µl of the mixture, prior to overnight incubation at 4°C. The wells were then washed 5 times with PBS.

- Detecting antibody

Mouse anti-NGAL antibody diluted at a 1:3000 dilution rate in 5% skim milk was used as detecting antibody. 100 µl of the mixture was added in the wells prior to incubation at 37°C for 1 hour. The wells were then washed 5 times with PBS.

- Enzyme-linked secundary antibody

Goat anti-mouse-Fc antibody was diluted at a 1:5000 rate in 100 µl of 5% skim milk and added in each well. The plate was then incubated for 1 hour at 37°C and the wells were then washed 10 times with PBS.

- Substrate addition

100 µl of TMB was added in each well and the plate was left 5 minutes in a dark room. The reaction was then neutralized with 50 µl of H_2SO_4 (2M).

- Revelation

The optical density of each well was then analyzed by spectrophotometry (wave length: 420-620nm).

The assays were repeated twice for each sample and the average OD was determined as final result. For more details concerning the optimized protocol, see Protocol 22 in Appendix 1.

1.4. Immunohistochemistry

1.4.1. Nature and origin of the samples

Samples were obtained by excisional biopsy from 43 female dogs admitted to Chung-Hsing University's veterinary medicine teaching hospital for clinical evaluation and treatment of mammary tumors during the years 2010 and 2011. None of these dogs had undergone any chemotherapy or radiotherapy prior to the mastectomy or nodulectomy. All patient data was recorded (final diagnosis, histological grade, lymph node metastase status and several more informations). The histological diagnosis was established by pathologists after hematoxylin and eosin staining and histological classification as well as malignancy grading followed the WHO classification of canine mammary tumor (Misdorp et al., 1976). For each dog, the slides containing tissue representing the major diagnosis were kept for IHC.

In total, 43 samples have been tested. 32 were malignant tumors (20 complex carcinoma, 11 simple carcinoma, 1 carcinosarcoma) and 11 were benign (8 benign mixed tumors, 2 complex adenoma, 1 simple adenoma).

IHC was performed on 4 µm sections of formalin-fixed, paraffin embedded tissue samples.

To compare the expression of MMP-9 and NGAL in healthy and cancerous mammary tissues, IHC assays were also performed on 13 healthy samples. These samples were obtained from non cancerous mammary glands according to histological diagnosis that had been excised at the same time as the tumor, when a full mastectomy was performed.

1.4.2. Immunohistochemistry protocol for NGAL

The IHC protocols and the reagent conditions greatly differ and depend on the tissue type, the target protein, the antibody, the reagents used... Prior to start the staining of the samples, different assays were tested in order to determine the optimal conditions for specificity, background and resolution of the signal.

The following protocol (Protocol 23 in Appendix 1) describes the conditions that were judged to be optimal. More information concerning the conditions that have been tested can be found in the Discussion section of this work.

- Deparaffinization and rehydration

The slides were first deparaffinated in two successive pre-heated (50°C) xylene solution bath, each one lasting 15 minutes in a 50°C incubator. They were then gradually rehydrated by first soaking them in two successive 100% ethanol bath (two times three minutes), one 95% ethanol bath (three minutes), one 75% ethanol bath (three minutes) and finally one 50% ethanol bath (three minutes).

- Antigen retrieval

The reagents used in tissue fixation can form protein cross-links that mask the antigenic sites of the sample, thereby giving false negative staining or lowering drastically its strength. Therefore a citrate based solution that can break the protein cross-links and unmask the epitopes in formalin-fixed and paraffin embedded tissue samples was used.
For that, the slides were soaked in a plastic container with 1L of Citrate Buffer and were heated at 800 watts for 20 minutes. The level of the buffer was regularly checked to make sure it was not excessively lowered because of evaporation.

The plastic container is then cooled down in a room-temperature water-bath for ten minutes.

- Circling

The slides were shaken dry, the non interesting tissue portions were discarded using a cotton swab, and the tissue section is circled with the *Dakopen.* This circle allows to use smaller quantities of reagents and antibodies in the next steps and helps preventing the tissue from drying. Slides were then washed three times with ddH2O to remove the extra ink.

- Blocking

Background or unspecific staining often occurs if the blocking step is omitted.

- Inhibition of endogenous peroxydase: endogenous peroxydase in the tissue can also degrade the substrate used in the revealing step, thereby giving an unspecific staining. A *3%* hydrogen peroxyde solution was applied on the tissue sections for 10 minutes followed by three ddH_2O washes.

- Blocking of the unspecific binding of the primary antibody: the primary antibody can also bind unspecifically, therefore giving a false positive signal. A *5%* Goat Serum solution was applied on the slides for 30 minutes followed by three ddH_2O washes.

- Primary antibody

The primary antibody was diluted at a 1:400 dilution rate with Dako REAL™ antibody diluant and approximately 300 µl is applied on the tissue sections for two hours. During that time, the IHC box is closed with a lid to avoid tissue dehydrating. Slides were then washed five times with ddH_2O.

- Secundary antibody

As Dako REAL™ EnVision™ HRP Rabbit/Mouse secondary antibody can detect both mouse and rabbit antibodies, it was thereby used for both NGAL and MMP-9 IHC. It was applied on the tissue sections for 30 minutes and was followed by three washes of ddH_2O.

- Substrate

To reveal the peroxydase action of the secondary antibody's peroxydase, Dako REAL™ DAB (3,3'-diaminobenzidine) diluted to 1:250 in Dako REAL™ Substrate buffer was applied for two minutes and fifteen seconds and was then washed off by three washes of ddH_2O.

- Counterstaining

Tissue sections were counterstained with hematoxylin for thirty seconds. The slides were then washed with ddH_2O and soaked in tap water for one minute. The alkaline status of the tap water makes the hematoxylin form an insoluble blue aluminium haematin complex that colors the nucleus in blue.

- Dehydration and mounting

The slides were dried in a 50°C incubator overnight and were mounted the next morning. They were dried at room temperature for two hours before being read.

1.4.3. Immunohistochemistry protocol for MMP-9

The IHC protocol for MMP-9 was very similar to the one used for NGAL. The only difference remained in the dilution of the primary antibody (1:100 instead of 1:400) and in the duration time of the DAB (3 minutes instead of 2 minutes 15 seconds).

1.4.4. Positive and negative controls

- Positive controls

For each batch of IHC staining, one slide known to be positive was included. These slides served as quality control for the whole batch. If it was judged to be of lesser quality than usual, all the slides of the corresponding batch would be discarded.

- Negative controls

In order to prove that the positive signal of the sample slides is specific, a negative control for each new batch was used. For this negative control the primary antibody was replaced by pre-immune serum.

1.4.5. Reading and scoring

Each slide stained using the optimized IHC protocol was analyzed with following parameters:

- Existence and specificity of the staining
- Cellular distribution of the staining
- Homogeneity of the staining throughout the tumor section

Additionally, the expression of the protein was determined semi-quantitatively by scoring:

- The intensity score (IS) of the staining (by comparison with the standard slide, judged as highly stained)
    - no staining: score 0
    - low : score 1
    - medium: score 2
    - high: score 3
- The proportion score (PS) of positive tumor cells
    - Less than 10%: score 1
    - 11-50%: score 2
    - 51-80%: score 3
    - More than 80%: score 4

The final score (FS) of a slide was the product of those two scores. A FS between 0-2 is considered negative, a FS between 3-7 is considered weakly positive and a FS between 8-12 is considered strongly positive. The score was evaluated by two different observers. If their opinions were different, the score would be discussed until an agreement was found (If no agreement could have been found, the slide would have been discarded from the study but this problem did not occur).

1.5. Statistical analysis

T-tests (with or without Aspin-Welsh correction) for the ELISA results, Fisher exact tests for the ELISA and IHC results and Spearman's correlation test to evaluate the correlation between MMP-9 and NGAL expression were all performed with GraphPad™ Prism software. All tests resulting in a $p < 0.05$ were considered significant.

2. RESULTS

2.1. Anti canine MMP-9 antibody

After the bleeding and the serum retrieval, the efficiency of the antibody was tested by Western-Blot on different samples. It was first verified that the mice serum could detect the recombinant MMP-9-Dist protein, prior to check its efficiency on commercial human MMP-9 and finally on canine mammary gland tissue homogenates.

2.1.1. Ability to detect the recombinant MMP-9-Dist

As seen on figure 17, the anti-histidine tag antibody, used as a positive control shows a signal at 22kDa which is the size of MMP-9-Dist. The sera taken prior to 2nd and 3rd boost can also detect target protein and with a signal strength that increases as the boost number raises. The fact that the pre-immune serum does not detect any protein serves as a negative control. Another protein (around 40kDa) seems to be strongly detected by the serum; this is due to the fact that the purity rate of the MMP-9-Dist used for this test was around 95% and other endogenous proteins from the host may have also induced an immune reaction.

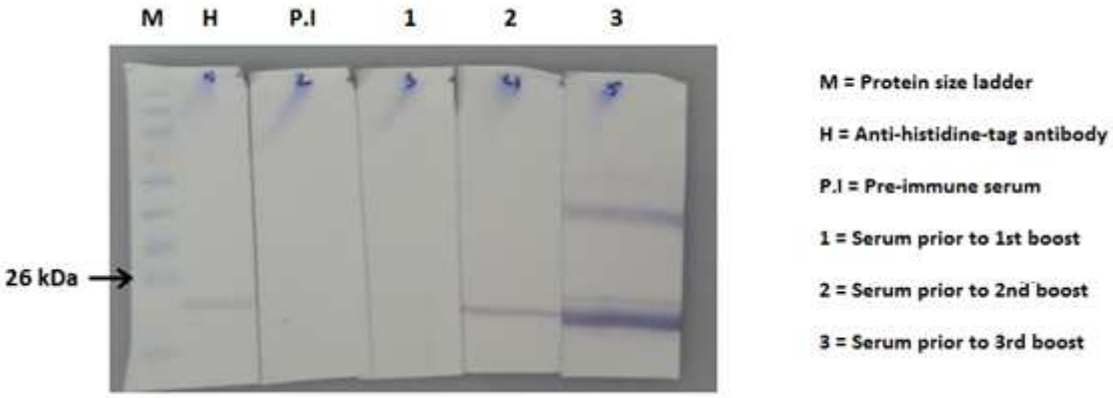


Figure 17: Verification of the mouse-2's serum immunized with recombinant MMP-9 by Western blot analysis

Recombinant MMP-9-Dist was resolved with SDS-PAGE and analyzed by Western blotting with various antibodies including anti-histidine tag (H), mouse pre-immunized serum (P.I), and the serum taken prior to 1st(1), 2nd(2) and 3rd (3) booster.

2.1.2. Ability to detect human MMP-9

As no commercial canine MMP-9 exists, capacity of the mouse serum to detect the commercial human MMP-9 (abcam™) was evaluated by western blot.

In this case, a negative result would not be interpretable as the antibody could have been specific enough for the canine protein but not to the human one. In this study, the antibody was indeed able to detect the human protein with a good efficiency (figure 18).

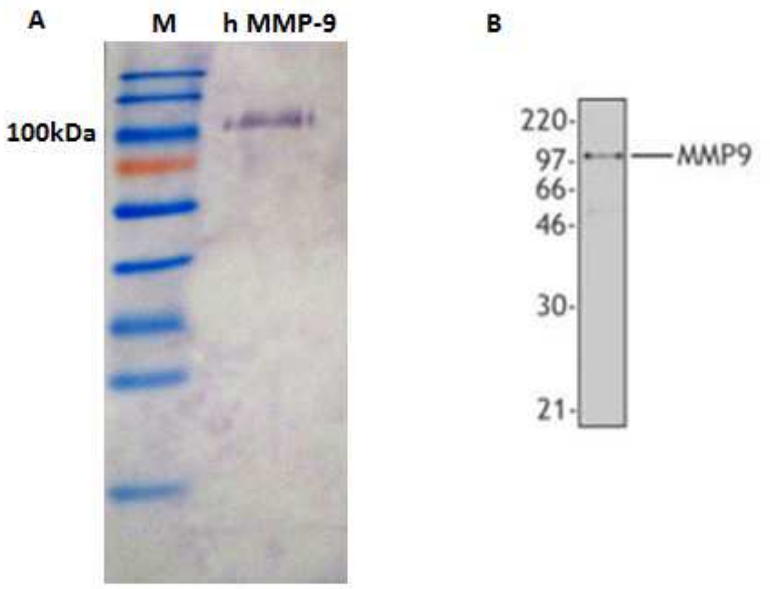


Figure 18: Detection of human MMP-9 by the serum of mouse 2

A: Commercial human MMP-9 was resolved with SDS-PAGE and processed for Western blot analysis with mouse 2 serum prior to the 3rd boost as primary antibody.
B: Expected size of the human pro-MMP-9 as announced by the product description

2.1.3. Ability to detect canine MMP-9

As mentioned earlier, although no commercial canine MMP-9 protein exists, the antibody efficiency was directly tested on mammary gland tissue homogenate. Two different samples were tested: mammary gland carcinoma and mammary gland adenoma homogenates.

A strong signal could be noticed at around 100kDa which corresponds to pro-MMP-9. The second signal, detected few kDa lower than pro-MMP-9 (approximately 90kDa) and of weaker intensity, corresponds to the expected size of the cleaved and active MMP-9 (figure 19).

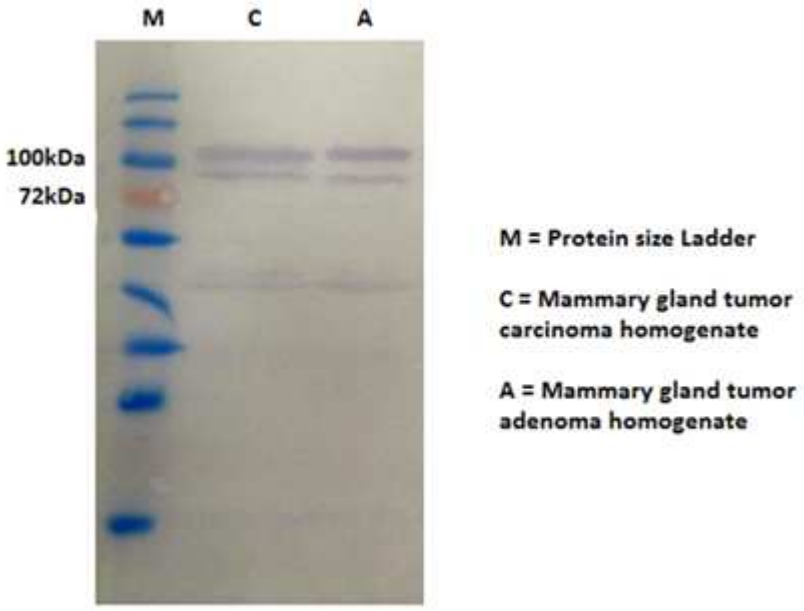


Figure 19: Verification of the antibody authenticity on mammary gland tumor tissue homogenates

Mammary gland tumor carcinoma homogenate (C) and mammary gland tumor adenoma homogenate (A) were resolved with SDS-PAGE and processed with Western blot analysis with mouse 2 serum prior to the 3rd boost as primary antibody.

2.1.4. Evaluation of relative titers antibody of mouse sera

An ELISA assay was performed using the recombinant MMP-9-Dist as the antigen and the mouse immunized serum as primary antibody. Both pre-immune (PIS) and final sera (FS) of all three mice were tested. Each one was tested at different dilutions, from 1:100 to 1:3200 in order to determine the relative antibody titer of the different sera. The endpoint titer was determined by calculating a cut-off (equaling to two times the mean optical density of the negative control) and by determining the antibody dilution that would have been necessary to obtain a result equal to the cut-off. The end point titer was calculated with GraphPad Prism® software.

The highest endpoint titer was obtained for mouse 2 at 1:5841 (table 1) that was then used for the immunohistochemical assays.

Table 1: Titration of the sera by ELISA (optical density)

Dilution	Mouse 1		Mouse 2		Mouse 3	
	PIS	FS	PIS	FS	PIS	FS
1:100	0.046	0.527	0.045	0.670	0.047	1.01
1:200	0.048	0.453	0.045	0.475	0.044	0.486
1:400	0.048	0.306	0.047	0.356	0.045	0.269
1:800	0.041	0.208	0.043	0.266	0.045	0.187
1:1600	0.041	0.132	0.049	0.228	0.045	0.115
1:3200	0.044	0.075	0.042	0.1015	0.044	0.064
Titer	-	1:2673	-	1:5841	-	1:2790

2.2. NGAL Sandwich ELISA

2.2.1. Blood samples

Because the standard curve, made by regression of the standard values, was not linear, and because the inter-assays variations were relatively high for standard samples as compared to the clinical samples, it was decided not to intrapolate the NGAL expression from the optical density (OD) values. The expression was thereby evaluated by comparing the different ODs observed for all the samples (table 2 and figure 20).

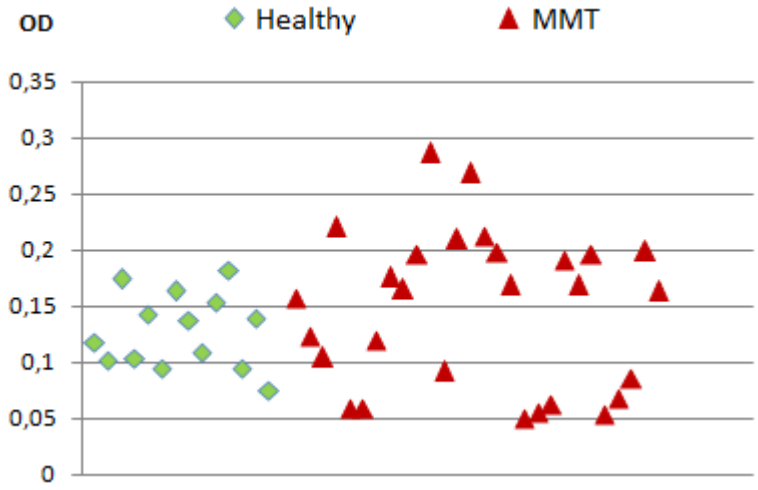
The average and median ODs of the healthy sera were respectively 0.128 and 0.127, barely lower than the average (0.147) and median (0.164) values observed for MMT samples.

Table 2: Optical density observed after NGAL sandwich ELISA performed on healthy and MMT sera

Healthy	MMT
0.075	0.050
0.095	0.053
0.095	0.056
0.102	0.058
0.103	0.059
0.109	0.062
0.118	0.067
0.137	0.086
0.139	0.092
0.142	0.104
0.153	0.120
0.163	0.123
0.174	0.157
0.181	0.163
	0.165
	0.168
	0.169
	0.175
	0.191
	0.196
	0.196
	0.198
	0.199
	0.209
	0.213
	0.220
	0.269
	0.286

	Healthy (n=14)	MMT (n=28)
Mean	0.128	0.147
Median	0.127	0.164
Standard deviation (Sd)	0.033	0.069
Extreme values	0.075 - 0.181	0.050 - 0.286
Cut-off = Mean + 2*Sd	0.193	

As the variances of these two sets of data were unequal, the Aspin-Welsh corrected T-test was performed on these values and obtained a p-value equal to 0.116, showing that there is no significant difference in the average serum NGAL expression between healthy samples and MMT samples.



<u>Figure 20</u>: Repartition of the optical density values observed after NGAL sandwich ELISA performed on healthy and MMT serum samples

A cut-off value was created by calculating the average of the healthy sample values added to two times the standard deviation of the values. The cut-off value resulted in 0.193, which gave the following contingency table (table 3).

<u>Table 3 : Serum NGAL expression contingency table after definition of the cut-off value (0,193)</u>

Optical density	Healthy	MMT
< Cut-off (0.193)	14	19
> Cut-off (0.193)	0	9

P=0.0155

Sensitivity=0.4242 / Specificity=1

Fisher's exact test applied to this contingency table shows a significant correlation between a serum NGAL concentration higher than the cut-off value and cancer disease status (p=0.0155), however the sensitivity of this test remains low (42%).

2.2.2. Urine samples

The average and median urine concentrations of NGAL were higher in MMT (0.097 and 0.086) than in healthy patients (0.048 and 0.048) (table 4 and figure 21); as the variances were unequal, the Aspin-Welsh corrected T-test was performed and a p-value equal to 0.0342 was obtained, suggesting that the difference is significant.

Table 4: Optical density observed after NGAL sandwich ELISA performed on healthy and MMT urinary samples

Healthy	MMT
0.044	0.042
0.046	0.048
0.048	0.061
0.049	0.076
0.053	0.096
	0.165
	0.189
	0.752

	Healthy (n=5)	MMT (n=8)
Mean	0.048	0.097*
Median	0.048	0.086
Standard deviation (Sd)	0.003	0.058
Extreme values	0.044 - 0.053	0.042 - 0.752
Cut-off = Mean+2*Sd	0.0541	-

*Note that the value at 0.752 was judged too extreme and was excluded from calculation

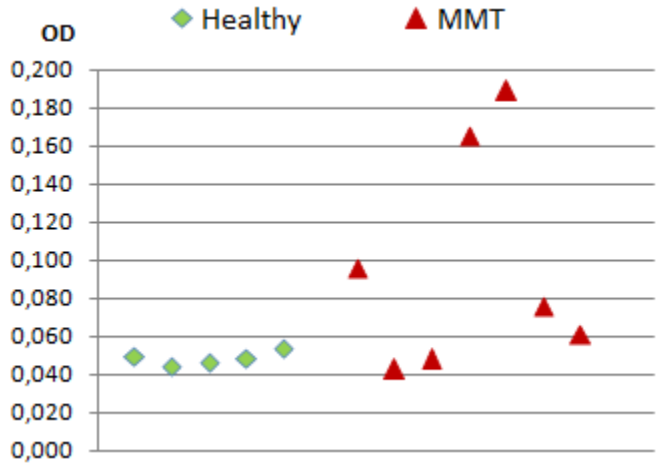


Figure 21: Repartition of the optical density values observed after NGAL sandwich ELISA performed on healthy and MMT urine samples

Note that the extreme value of (0.752) was not added on the graph

A cut-off value was defined as equal to (average + 2*Sd) of the healthy samples values. The cut-off value was thereby 0.0541 and resulted in the contingency table presented as table 5.

Table 5: Urinary NGAL expression contingency table after definition of the cut-off value (0,0541)

Optical density	Healthy	MMT
< Cut-off (0.0541)	5	2
> Cut-off (0.0541)	0	6

P=0.0163

Sensitivity=1 / Specificity=0.75

The correlation between NGAL concentration in urine higher than the cut-off and cancer disease status was significant (Fisher exact test, p=0.0163). The cut-off showed an excellent sensitivity and an average specificity (100% and 75%).

2.3. NGAL Immunohistochemistry results

2.3.1. Efficiency of the staining

All 43 tumor samples slides and all 13 healthy tissue slides were immunohistochemically stained using the protocol described in the material and method section. Most of the samples, even tissue sections originating from healthy mammary glands, showed at least a few luminal epithelial cells positive for NGAL and MMP-9, whereas the negative control performed with pre-immune serum showed no positive staining (figure 22).

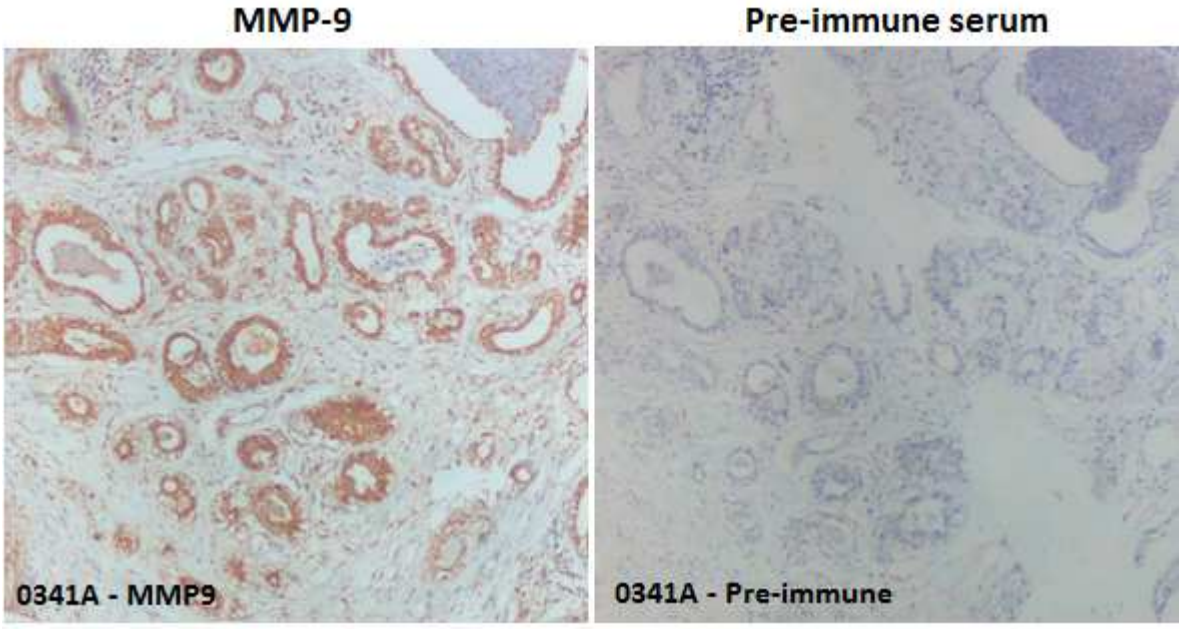


Figure 22: Comparison of tissue labeling with home-made anti MMP-9 antibody and pre-immune serum as a negative control

Expression of NGAL and MMP-9 was demonstrated in all the tumor samples. Marked differences between staining were most often noted not in the proportion of positive cells but rather in the intensity of expression of the studied proteins (figure 23). For both proteins, the staining was almost exclusively in the cytoplasm of luminal epithelial cells, but some rare stromal cells sometimes showed positivity.

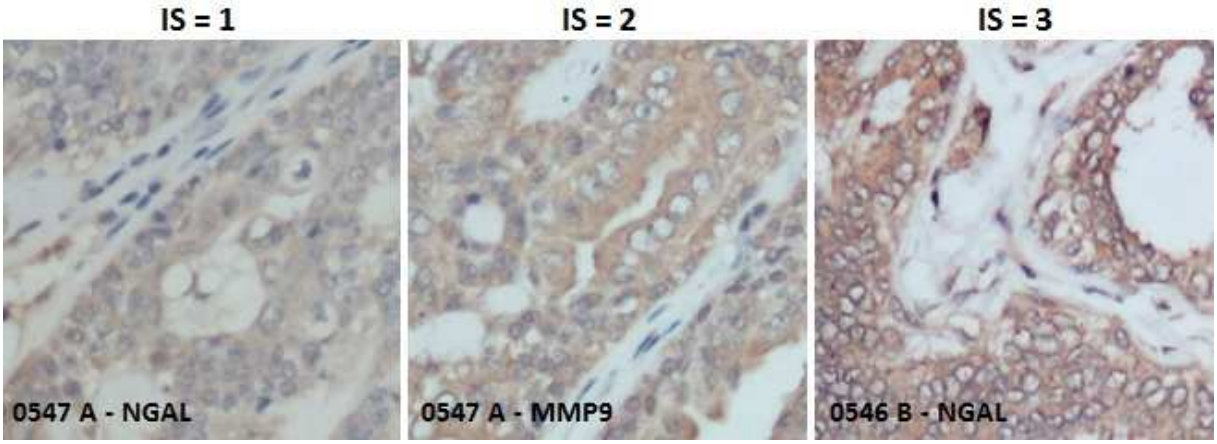
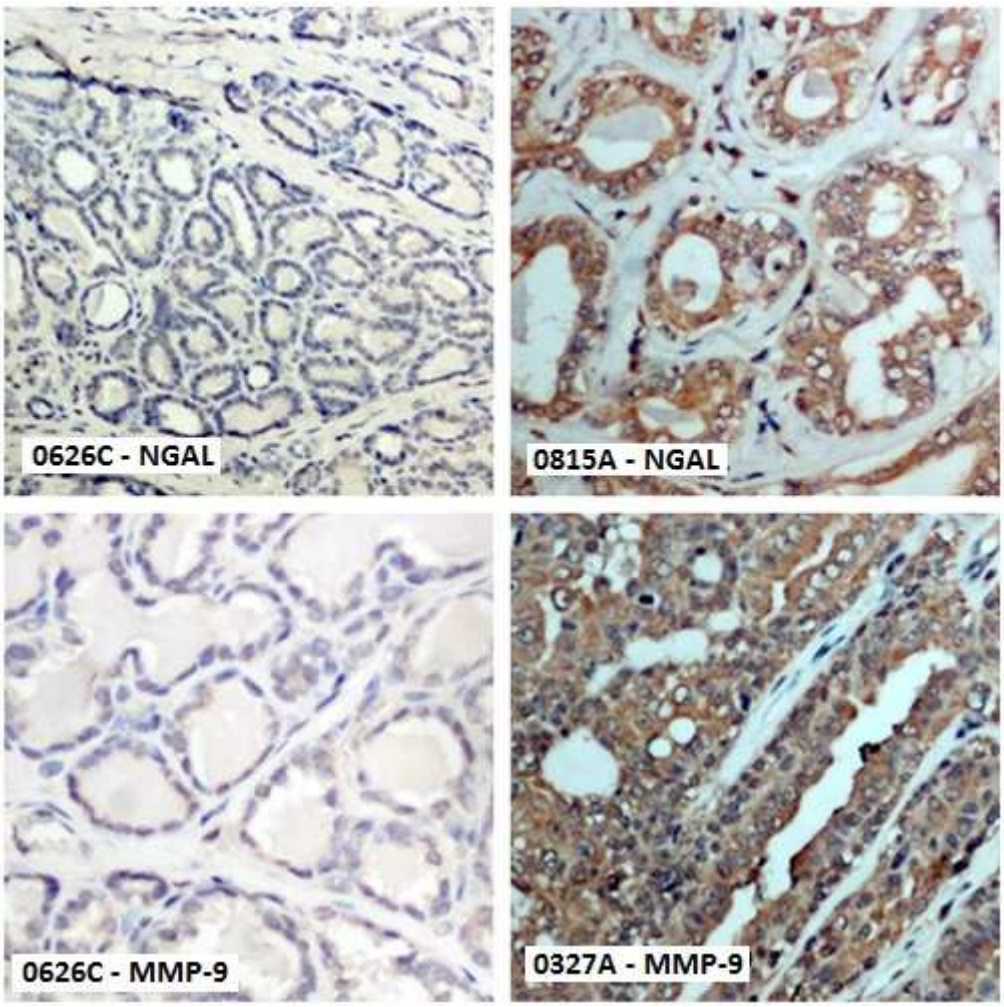


Figure 23: Intensity score graded between 0 (no staining) and 3 (strong staining)

2.3.2. Correlation between cancer status and MMP-9 and NGAL expression

Expression of NGAL (table 6) and MMP-9 (table 7) were determined semi-quantitatively in healthy tissues, tissues from benign tumors and tissues from malignant tumors. For MMP-9, most of healthy samples were negative (84.6%) and few were weakly positive (15.4%), whereas all tumor samples, either benign or malign, showed at least weakly positive (figure 24). For NGAL, the majority of healthy glands (61.5%) were negative; however the proportion of healthy samples to be weakly positive was much higher (38.5%) than for MMP-9 staining. For both proteins, no healthy control was noted as strongly positive.

The Fisher exact test was used to analyze differences in expression of MMP-9 and NGAL between healthy samples and tumoral ones. By grouping the weakly and strongly positive results together, a significant correlation between the expression of both proteins and the tumoral status of the tissue was recorded (table 8 for NGAL and table 9 for MMP-9).



<u>Figure 24</u>: NGAL and MMP-9 immunostaining of healthy and tumoral mammary glands

0626C is a healthy gland whereas 0815A is a grade III simple carcinoma and 0327A is a grade II complex carcinoma.

Table 6: NGAL expression determined semi-quantitatively by immunohistochemical staining in tumoral cells

Final score (FS)	Healthy	Benign tumor	Malignant tumor
Negative (0-2)	8 (61.5%)	0	0
Weakly positive (3-6)	5 (38.5%)	5 (45.5%)	8 (25%)
Strongly positive (8-12)	0	6 (54.5%)	24 (75%)

Table 7: MMP-9 expression determined semi-quantitatively by immunohistochemical staining in tumoral cells

Final score (FS)	Healthy	Benign tumor	Malignant tumor
0-2	11 (84.6%)	0	0
3-6	2 (15.4%)	7 (63.6%)	9 (28.1%)
8-12	0	4 (36.4%)	23 (71.9%)

Table 8: Differences in NGAL expression between healthy samples and benign or malignant tumor samples

	Healthy	Benign tumor	Malignant tumor
Negative (0-2)	8	0	0
Positive (3-12)	5	11	32
		p=0.0017 Sensitivity: 1 Specificity: 0.62	p<0.0001 Sensitivity: 1 Specificity: 0.62

Table 9: Differences in MMP-9 expression between healthy samples and benign or malignant tumor samples

	Healthy	Benign tumor	Malignant tumor
Negative (0-2)	11	0	0
Positive (3-12)	2	11	32
		p<0.0001 Sensitivity: 1 Specificity: 0.85	p<0.0001 Sensitivity: 1 Specificity: 0.85

Table 10: Differences in NGAL expression between benign and malignant mammary gland tumors in dogs

	Benign tumor	Malignant tumor
Weakly Positive (3-6)	5	8
Strongly Positive (7-12)	6	24

p=0.1845

Sensitivity: 0.75 / Specificity: 0.45

Table 11: Differences in MMP-9 expression between benign and malignant mammary gland tumors in dogs

	Benign tumor	Malignant tumor
Weakly Positive (3-6)	7	9
Strongly Positive (7-12)	4	23

p=0.0422

Sensitivity: 0.72 / Specificity: 0.64

2.3.3. Correlation between NGAL and MMP-9 expression and the malignant/benign tumoral status

The NGAL expression could not be significantly correlated with the malignant or benign status of the tumor (table 10). Even though malignant tumors tended to have a stronger staining than the benign ones, the Fisher test gives a non statistically significant result (p=0.18).

However, MMP-9 expression seems to be significantly correlated with the malignancy status of the gland tumor (table 11). However, both the sensitivity and the specificity remain moderate (sensitivity = 72% and specificity = 64%).

2.3.4. Expression level and other prognostic factors

For both proteins, their expression could not be significantly associated with the histological type of the malignant tumors. Concerning the histological grading of the tumors, NGAL's expression could not be correlated with the grade. Differences in MMP-9 expression between grade I and grades II or III were not proven significant. The small number of samples, especially for grade III (n=4) is an obstacle to the statistical analysis. However, if grade II and III are grouped together, there is a significant difference in MMP-9 expression between grade I and grades II-III (p=0.044, table 12).

Table 12: MMP-9 expression according to histological grade of malignant tumors

Histological Grade	I	II	III
Weakly positive	4 (50%)	2 (14.3%)	0 (0%)
Strongly positive	4 (50%)	12 (85.7%)	4 (100%)

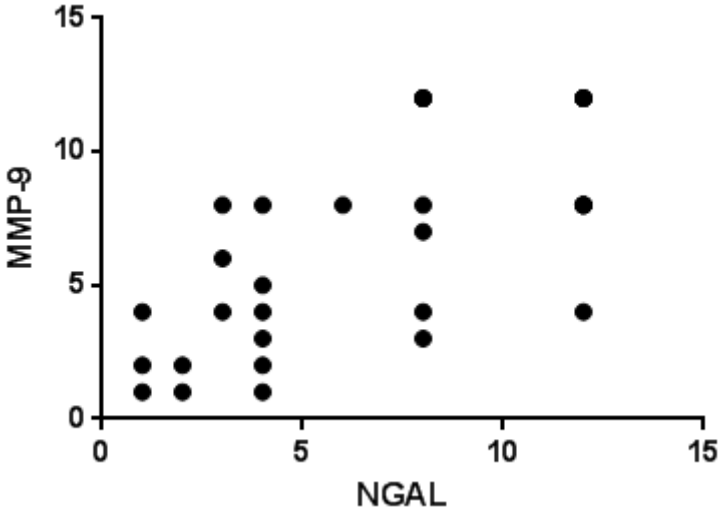
The difference between grade I and grade II and III grouped together is significant (p=0.044)
Sensitivity: 0.89 / Specificity: 0.50

No significant correlation was found between the expression of the two proteins and the age of the patient, the number of tumors, the neutered status, the survival time, and the lymph node metastasis status.

2.3.5. Correlation between NGAL and MMP-9 expression in mammary gland tissues

Because NGAL and MMP-9 are both efficient tissue biomarker of mammary gland tumor disease in humans, and because they have been shown to form a complex and to interact with each other, especially as NGAL protects MMP-9 from degradation, the correlation between the expression of the two proteins was investigated (figure 25).

Spearman correlation test showed that the expression of the two protein are highly correlated (p=0.0001) and exhibit parallel variations (r=0.67).



<u>Figure 25</u>: Correlation between MMP-9 and NGAL expression in mammary glands considering the whole cohort (healthy and tumoral samples)

Note that some points, especially in the diagonal (1,1; 2,2; 3,3; 4,4; 6,6; 8,8 and 12,12) correspond to multiple samples

It is worth noting that 36/56 (64%) of the samples belonged to the same category (negative, weakly positive, strongly positive) for both MMP-9 and NGAL expression.

3. DISCUSSION

3.1. Production of the MMP-9 antibody: optimization of the protocol

The first serious problems encountered in the production of the antigen started at the steps of protein expression and purification. A small scale expression had been performed in order to determine the best conditions for the expression protocol. The results obtained indicated that an incubation at 37°C for 9 hours after IPTG induction was the optimal conditions, resulting in a massive expression of the MMP-9-Dist.

However, several problems occurred at this stage:

- Another protein (about 4kDa heavier than MMP-9-Dist) was massively expressed by the BL21 bacteria during incubation.
- This contaminant protein became problematic during the protein extraction step, when it was observed that it could bind very effectively to the nickel charged sepharose and contaminated the elution samples.
- Furthermore, the overall binding efficiency of the protein extraction procedure was extremely low (figure 26).

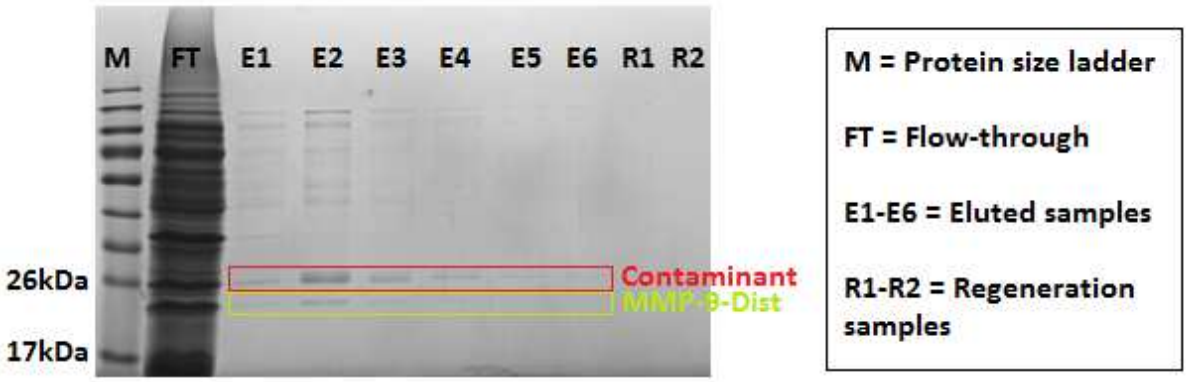


Figure 26: SDS-PAGE after protein extraction with the Ni^{2+} charged sepharose according to the first protocol

- Large scale expression conditions: 37°C for 9 hours after IPTG induction.
Protein extraction conditions: 6M urea, 0.5M NaCl, binding at room temperature.
Note the low efficiency of the binding as most of the MMP-9-Dist stayed in the flow-through.

3.1.1. Contaminant protein

The contaminant protein was noticed to be absent in the no-induction samples and was highly expressed after IPTG induction when large scale expression was done at 37°C for 9 hours, suggesting a leaky expression or post-translational modifications of the recombinant protein. The later hypothesis is strengthened by the fact that it also showed very weakly positive for the his-tag when tested by Western Blot with an anti-his antibody.
To solve this issue, temperature and time were modified during large scale expression in order to verify if they impacted the expression of MMP-9-Dist and of the contaminant protein.

After multiple tries, it was found that changing the large scale expression conditions from 9 hours at 37°C to 24 hours at 25°C cut down the yield of the recombinant MMP-9-Dist protein by about half but also induced an extremely low expression of the contaminant protein. It was decided to use these new conditions for the rest of the study.

3.1.2. Binding efficiency during chromatography

To increase the low efficiency of the protein binding by the charged sepharose beads, probably due to the protein's three dimensional structure which may hide the histidine-tag, stronger denaturing conditions (particularly by changing the buffers concentration in urea, in NaCl, their pH or temperature) were explored. in particular its concentration in urea, in NaCl, its pH or its temperature. An interesting improvement when the urea was increased from 6M to 8M and when the NaCl concentration was doubled from 0,5M to 1M was obtained. A small increase was also noticed when the binding step was performed at 4°C. By trying these three conditions together, the binding efficiency was greatly improved.

By combining this new protein extraction protocol with the optimized large scale expression protocol, the overall protein production and purification was greatly improved (figure 27).

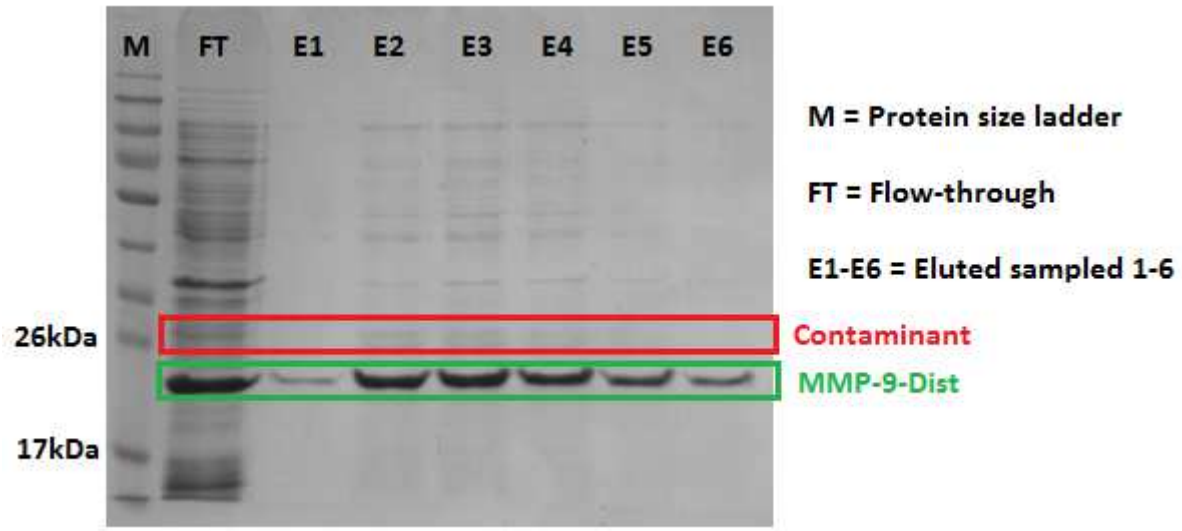


Figure 27: SDS-PAGE after protein extraction with the Ni^{2+} charged sepharose with the modified protocol of large scale expression and chromatography binding

Large scale expression conditions: 25°C for 24 hours after IPTG induction.
Protein extraction conditions: 8M urea, 1M NaCl, binding at 4°C.
Note the good efficiency of the binding as the eluted samples are rich in MMP-9-Dist. The contaminant protein is very poorly expressed and MMP-9-Dist is the major component in the samples.

3.1.3. Impact on immunization

These two issues highly affected the yield and purity rate of the produced protein used for the prime-injection and the first booster injected to the mice. With the optimized expression and extraction conditions, it was possible to produce large amount of high purity rate antigen for the following boosters.

3.1.4. Prospect to establish a home-made MMP-9 sandwich ELISA test

Because of the difficulties experienced during the purification of MMP-9-Dist, it was not possible to harvest enough antigens to immunize both mice and rabbits. As a sandwich ELISA assay requires two different antibodies that originated from two different species it was not possible to make it with only the home-made mouse antibody.

After the issues concerning the antigen purification have been resolved, enough antigens were produced to allow immunization of two rabbits. However the delay needed for the rabbits to produce the antibody did not allow us to harvest the antibody and establish a home-made sandwich ELISA test before the end of the study.

3.2. Expression of NGAL in the urine and serum

3.2.1. Limits and reproducibility

- Representativity of the samples

The aim of this study was to create a preliminary methodological study of the evaluation of the NGAL expression in the urine and blood samples of female dogs suffering from mammary gland tumors. Though the number of blood samples (28 MMT samples and 14 healthy samples) was consistent enough, the low number of urine samples (8 MMT samples and 5 healthy samples) that were tested is the main limit of this preliminary study. As the results obtained seemed encouraging, it would be interesting to repeat this assay on a much larger number of urine samples.

- Unreliable standard curve

The original aim of this assay was to determine the exact concentration of NGAL in the different samples by comparing their OD values to a standard curve established using a set of recombinant NGAL standards.

However it was difficult to use this standard curve to intrapolate the NGAL concentration in the samples, mainly for two reasons:

- The reproducibility of the standard curve was moderate. Whereas the values found for clinical samples was very stable from one assay to another, it was not the case for the standard samples as differences up to about 25% could be noted from one ELISA test to another.
- The standard curve was not linear. It is possibly due to the continuous loss of proteins during serial dilutions. Attempts were made to dilute the recombinant NGAL in PBS containing 5% skim milk rather than in PBS. However, results were barely improved. Low retention tips and microcentrifuge tube can be used to avoid the trace amount protein remains in plastic wares.

- Reproducibility of the assays

Because of the number of blood samples (n=42) being higher than the number of triplicated wells available on one ELISA plate, all samples could not be assayed on the same plate. However, the assays were repeated two times and the high similarity found in the measured ODs showed that the inter-assays bias was neglectable.

3.2.2. Serum and urine NGAL concentrations as markers of mammary gland tumor affection

Even though serum NGAL concentrations tended to be higher in dogs suffering from a MMT than in healthy controls, the difference was not big enough to be significant, unlike the results found in human breast cancer. However, it must be noted that even in assays carried on human patients, the difference of the serum NGAL concentrations, though significant, was not very important. Provatopoulou et al for instance, found an average NGAL concentration in serum of DCIS (ductal carcinoma in situ) patients of 80.0 ng/ml (+/- 22.5) , and in serum of IDC (invasive ductal carcinoma) patients equal to 87.4 ng/ml (+/- 28.1), these concentrations being barely higher than in healthy controls (70.7 ng/ml +/- 17.4). Considering the narrow difference of the two mean concentrations, and the relatively large standard deviations, a very large number of both cancerous and healthy samples would be required to prove the significance of the increase. Furthermore, the significant result obtained after application of a cut-off value confirms that NGAL could be an interesting diagnostic biomarker.

In correspondence with the findings made in human patients, NGAL urinary concentrations were significantly correlated with malignant mammary tumor in the sample panel. These results suggest that NGAL could be a valuable urinary biomarker of cancer status in female dogs. However as mentioned earlier, it would be interesting to investigate this on a larger scale with a bigger number of samples and to study its correlation with other factors such as lymph node metastasis status, disease-free survival time or histological grade for example, which was not possible here due to the small quantity of samples.

3.3. Expression of NGAL and MMP-9 in mammary tissues

3.3.1. Optimization of the protocol

The main conditions that have been tested are:

- Antigen retrieval: power of the microwave and duration. Power was tested from 600 to 1000 watts, 800 watts seemed to be the best condition as higher power sometimes caused degradation of the fragile tissue sections. For similar reasons, the duration selected was 20 minutes.

- Blocking: the first reagents used to block were the commercial ones from Dako™'s kit. It was found to be slightly less effective than 3% H_2O_2 and 5% goat serum as the use of these two reagents seemed to give lower backgrounds.

- Primary antibody dilution and duration: many different dilutions have been tested (from 1:50 to 1:800). Increased dilutions affected the strength of the positive staining negatively. The optimal dilutions provide correct staining intensity and allow to minimize the antibody consumption; they were 1:400 and 1:150 for NGAL and MMP-9 IHC, respectively. Also the following application durations have been tried:
    - at room temperature: 1 hour, 2 hours, 4 hours
    - at 4°C: overnight

  For both antibody, an incubation of two hours at room temperature, as the staining was slightly better than one hour, and similar to the longer durations, was retained.

- DAB dilution and duration were the hardest conditions to optimize. During the first trials, three different dilutions (1:100, 1:250, 1:500) were assessed. The reaction was stopped after 30 seconds and the slide was briefly observed under microscope, and gradually DAB was added every 30 seconds for evaluating the revelation step. When no improvement in the intensity of the positive signal was noted, or when the background started to lose its clarity, the precedent DAB conditions (dilution and exposure duration) were retained. These steps had to be repeated several times before decision was taken to use 1:250 dilution for 2 minutes 15 seconds for NGAL and 3 minutes for MMP-9.

3.3.2. Limits and reproducibility

- Representativity of the tumor samples

The aim of this study was set to evaluate the expression of the two proteins and not the exact quantification of their expression in mammary gland tumor; the number of samples was voluntarily restricted. However, it must be pointed that the proportion of tumor types is not representative of the epidemiology of mammary tumor pathology in dogs: benign tumors represent only 26% of the total number of samples, much lower than the real proportion. The small number of benign tumors analyzed (11) also impacted the statistical analysis and it would be interesting to test more samples. Furthermore, the low number of samples in each sub-category of different prognostic factors unabled us to make any conclusion about the eventual relation between the protein's expression level and these factors.

- Representativity of the healthy samples

Unlike the other studies performed on MMP-9 IHC, it was not possible to get normal mammary glands after euthanasia of dogs showing no palpable mammary gland masses. For the healthy controls, glands that were normal as diagnosed by histological examination from dogs that had undergone full mastectomy were used. The healthy controls are thereby healthy glands from dogs suffering from another mammary gland tumor. As known, the different mammary glands are heavily linked, in particular by the aterious/veinous and lymphatic networks. The existence of a mammary gland tumor on one mammae may also impact the MMP-9 or NGAL expression in healthy glands of another mammae. This could explain that the present results for MMP-9 expression are slightly different from ones reported in earlier studies. Even though, the expression in the healthy samples remained in majority negative, and only two of them showed low positive MMP-9 expression, whereas healthy samples were all negative in the assays performed by Hirayama et al in 2002.

- Reading and scoring relevancy

The slide reading and scoring were performed by two different observers. When the grading was different, the final score was discussed until both viewers agreed on a compromise. However, the two observers were not expert pathologists, and as IHC scoring is a complicated and somewhat subjective technique, the small experience of the viewers might have sometimes affected the relevancy of the scoring.

3.3.3. MMP-9 and NGAL as tissue markers of mammary gland tumors

- MMP-9 as a marker of mammary gland tumor affection, malignancy status and histological grades

The MMP-9 expression was a valuable tissue marker in the detection of mammary gland tumoral cells. In line with the studies previously performed by Hirayama et al (2002) and Santos et al (2012), it was also confirmed in the present study that canine MMP-9 expression in the tissue was significantly correlated not only with the tumoral status, but also with the malignancy status of the tumor.
Furthermore, the MMP-9 expression has significantly differed between tumors of grades I and tumirs of grade II and III. Out of the three groups, grade I samples showed the lowest MMP-9 expression but the difference with grade II was not significant, probably because of the low number of samples from each category of histological grade.

Unlike the recent findings of Santos and coworkers, the MMP-9 expression failed to relate to any other prognostic factors. The low number of lymph node metastasis positive samples (5) and the recentness of the biopsies (half of the tumors were excised less than a year ago) were obstacles in the demonstration of MMP-9 expression's correlation with metastasis or decrease of survival time.

- NGAL expression in correlation with cancer disease status

This is the first study to investigate the expression of canine NGAL in mammary gland tumor tissues. The assays show, in accordance with the results found on human patients by Bauer et al in 2008, that canine NGAL expression is significantly increased in tumoral tissue compared to healthy controls. However, the difference of NGAL expression between benign and malignant tumors was not significant and, unlike Bauer et al, no correlation between NGAL's expression and prognostic factors such as lymph node metastases status, or poor histological grade was found. The lack of significance may inherit from the low number of samples with known positive lymph node metastases status (5), or with the low number of histological grade I (8) and III (4) tumors. It would be interesting to perform immunohistochemistry on a very large number of tumor samples in order to have enough samples for each subcategory of commonly used prognostic factors.

3.4. Future prospects

In a next future, Chung Hsing University's Graduate Institute of Microbiology and Public Health will aim to investigate the expression of MMP-9 in urine and blood samples by using a home-made sandwich ELISA system using the antibodies produced in this study. Two rabbits were immunized at the end of this study. Once their serum shows a satisfying antibody titer, it will be attempted to establish a home-made sandwich ELISA protocol to evaluate the expression of MMP-9 in the blood and urine samples of female dogs suffering from mammary gland tumors.

An assay to detect the MMP-9/NGAL complex in the urine or blood will also be attempted, as it was showed in human breast cancer that the complex of the two proteins was a very interesting marker of breast cancer status and prognostic factors (Provatoupoulou et al., 2009). Using the home-made canine NGAL antibody as coating antibody and the home-made canine MMP-9 one as detecting antibody could give interesting results.

The significant increase in NGAL expression in urines suggest that if studies performed on a larger number of samples tend to confirm this observation, NGAL could be a valuable marker by a non-invasive technique of mammary gland tumor diagnosis. However, it would serve better as an exclusion test as the specificity is much higher than the sensitivity. A larger number of samples would also mean it would be possible to investigate the possible correlation between NGAL and prognostic factors such as histological grade or lymph node metastases.

The results obtained in the immunohistochemical assays are encouraging. It shows not only that NGAL and MMP-9 are interesting tissue markers of mammary gland tumor diseases but it also suggest that, in agreement with the findings made in human medicine (Fernàndez et al., 2005), NGAL and MMP-9 may possibly play an interesting role in canine tumor progression, particularly MMP-9 since it was correlated with histological type and malignancy status.

If more studies confirm the role of NGAL and MMP-9 in tumors, a pertinent question is – could therapeutic inhibition of these proteins slow down tumor progression, and if so, how could they be targeted therapeutically? Currently, there are no specific inhibitors of NGAL (Chakraborty et al., 2012). However matrix metalloproteinase inhibitors (MMPIs) have been developed as therapeutic drugs against cartilage and bone breakdown in rheumatoid arthritis (Ahles et al., 1986). MMP-9 activation and expression involves many steps (such as gene transcription, RNA translation, secretion of the zymogen, activation of the zymogen and degradation) and most of them are modulable throughout pharmacological intervention. Many different MMPI have thereby been developed and some have been tested in breast cancers. Marimastat pre-clinical studies for example were associated with a significant reduction in number and size of metastatic foci in treated versus control animals (Wojtowicz-Praga et al., 1997). BAY 12-9566 is another potent inhibitor of MMP-9 (as well as MMP-2 and -3) and induced tumor growth inhibition against human breast tumor xenografts (Nozaki et al., 1998). It must be noted however that despite the fact that the enthusiasm for the use of MMPIs to

treat cancer motivated a large implication from pharmaceutical industry, the first clinical trials results were not as encouraging (Pavlaki et al., 2003).

Furthermore, canine mammary gland tumors and human breast cancers share many similarities. They share the same histological origins, the same types of regional and general metastatic extensions, a similar age of appearance (proportionally compared to the life expectancy of the two species). It is also the most common type of cancer found in both species and carcinoma is the main histological type (Hill et al., 2005). The fact that the present MMP-9 and NGAL immunohistochemical results share similarities with those found in human medical research may suggest the use of canine mammary gland tumor as a spontaneous model for human breast cancers in the investigation of therapeutic tools through modulation of the MMP-9 and NGAL activities. Currently, NGAL and MMP-9 are mostly tested on mice, but dogs show several advantages compared to the murine models: the gene homology between dogs and humans is higher and the gene families implicated in tumoral lesions of the mammary gland in dogs and humans have been showed to be highly similar (Uva et al., 2009). Unlike laboratory mice, dogs also share the same environment as humans and are thereby under the impact of the same environmental carcinogenous factors.

CONCLUSION

Mammary gland tumors represent one of the most studied group of tumors in canine veterinary research, mostly because of its high prevalence. The investigation of proteins that are overexpressed in tumoral cells is essential as not only can they sometimes be used as biomarkers, but they may also give interesting therapeutic solutions if they are known to have pro-cancerous effects.

We inspired ourselves from the studies made in human medicine, which have shown several times an undeniable correlation between the expression of both matrix metalloproteinase 9 (MMP-9) and neutrophil gelatinase associated lipocalin (NGAL) and the cancerous status. We have aimed to evaluate the expression of the two proteins in the same types of samples as those studied from human patients (urine, blood and mammary gland tissue samples).

Serum and urinary concentrations of canine NGAL from mammary gland tumor patients were evaluated for the first time by home-made sandwich ELISA assay while its tissue expression was evaluated by immunohistochemistry using home-made rabbit antibody. This is also the first study reporting the production and use of home-made anti canine-MMP-9 antibody for immunohistochemical assays.

The NGAL and MMP-9 expressions were correlated in mammary gland tissues, and both were shown to be significant tissue markers of tumoral status. Furthermore, MMP-9 was shown to be correlated with some prognostic factors such as malignancy status or histological grade. Furthermore, serum and urinary NGAL concentrations were higher in dogs suffering from malignant mammary tumor than in control dogs.

These results are similar to the findings in human breast cancer research and confirm the similarities between the mammary gland cancers between the two species. They also suggest that matrix metalloproteinase inhibitors (or possibly NGAL inhibitors) could be interesting to investigate as therapeutic agents against canine mammary gland tumor progression and metastasis.

APPENDIX

APPENDIX 1: PROTOCOLS

Protocol 1: RNeasy® mini kit for RNA extraction (Qiagen®)

Cell lysis	1. Extract 0.2 g of testicule tissue with a surgical blade 2. Pound the tissue using a mortar and pestle and pour liquid nitrogen to obtain cell lysis 3. Resuspend with 200 µl of PBS
Re-suspension	4. Put the 200 µl of sample in a new tube and add 500 µl of *RLT Buffer* and 700 µl of 70% ethanol
Lysis	5. Add 200µl of *PD2 Buffer* and mix gently (do not vortex)
Neutralization	6. Add 300µl of *PD3 Buffer* and mix gently (do not vortex) 7. Centrifuge at 14,000 x g for 3 minutes
RDNA Binding	*8.* Place the *PD Column* in a 2 ml *Collection Tube* 9. Add the supernatant from step 6 to the *PD Column* and centrifuge at 14,000 x g for 30 seconds and discard the flow-through
Wash	10. Add 400 µl of *W1 Buffer* into the *PD Column* 11. Centrifuge at 14,000 x g for 30 seconds and discard the flow-through 12. Add 600 µl of *Wash Buffer* (ethanol added) into the *PD Column* 13. Centrifuge at 14,000 x g for 30 seconds and discard the flow-through 14. Centrifuge at 14,000 x g again for 3 minutes to dry the column matrix
RDNA Elution	15. Transfer the dried *PD Column* to a new centrifuge tube and add 50 µl of *DEPC treated water* into the center of the column matrix and let stand 2 minutes 16. Centrifuge at 14,000 x g for 2 minutes to elute the RNA

The different buffers are kit contents and their exact composition is not detailed in the handbook. *DEPC treated water*: ddH_2O treated with 0.1% diethylpyrocarbonate and incubated overnight at 37°C prior to being autoclaved. This treatment inactivates RNase enzymes and protects the extracted RNA from degradation.

Protocol 2: Superscript III Supermix Kit for Reverse Transcription (Invitrogen™)

Mixing	1. In a PCR tube mix: -5 µl of *RT Reaction mix* -1 µl of *RT Enzyme mix* -2 µl of RNA sample -2 µl of *DEPC treated water*
Reverse transcription	2. Set the PCR machine for 1 cycle: -25°C for 10 minutes (enzyme activation) -50°C for 30 minutes (priming and extension) -85°C for 5 minutes (terminating) -chill on ice

Protocol 3: Amplification of the insert by PCR (Phusion™ DNA polymerase kit)

Mixing	1. In a PCR tube mix: -5 µl of 5xBuffer -2 µl of 2.5µM dNTPs mix (Protech™) -2.5 µl of 5µM F-Primer -2.5 µl of 5µM R-Primer -0.0625 µl of Phusion™ DNA polymerase -1 µl of template cDNA -11.75 µl of ddH2O
Amplification	2. Set the PCR machine as follows: -98°C for 30 seconds (denaturing) -35 cycles: -98°C for 10 seconds (denaturing) -62°C for 30 seconds (annealing) -72°C for 30 seconds (extension) -72°C for 10 minutes (final extension) -4°C

Protocol 4: 1% agarose gel

Gel preparation	1. Mix: -0.3 g of agarose -30 ml of *TAE Buffer* 2. Heat with a microwave oven until it boils 3. Add 2 µl *Nucleic Acid Stain* 4. Wait for the gel to cool down and solidify at room temperature 5. Insert 5 µl of sample in the well
Gel running	6. Run horizontal electrophoresis at 100V for 25 minutes
Revelation	7. Use an UV transilluminator to visualize the fluorescent DNA bands

TAE Buffer: Tris-Acetate EDTA
Nucleic Acid Stain: Healthview™ Nucleic Acid Stain

Protocol 5: pET-24a(+) and cDNA insert digestion by *Nde I* and *Xho I* (New England Biolabs® kit)

Mixing	1. In a PCR tube mix: -6 µl of ddH_2O -2 µl of *NE Buffer 4* 10x -2 µl of *BSA* 10x -1 µl of *Nde I* -1 µl of *Xho I* -8 µl of pET-24a(+) or cDNA sample
Digestion	2. Leave in 37°C incubator for 2 hours

NE Buffer 4: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol

Protocol 6: Gel DNA Fragments Extraction Kit (Geneaid™)

Gel dissociation	1. Excise the agarose gel containing the relevant DNA and remove any extra agarose to minimize the size of the gel slice (maximum 300 mg) 2. Transfer the gel slice to a 1.5 ml microcentrifuge tube 3. Add 500 µl of *DF Buffer* and mix by vortex 4. Incubate at 60°C for 15 minutes and cool down to room temperature
DNA binding	5. Transfer 800 µl of the sample mixture to the *DF Column* furnished in the kit 6. Centrifuge at 6,000 g for 30 seconds and discard the flow-through
Washing	7. Add 400 µl of *W1 Buffer* 8. Centrifuge at 6,000 g for 30 seconds and discard the flow-through 9. Add 600 µl of *Wash Buffer* and let stand for 1 minute 10. Centrifuge at 6,000 g for 30 seconds and discard flow-through 11. Centrifuge again for 3 minutes to dry the column matrix
DNA Elution	12. Add 50 µl of *Elution Buffer* and let stand for 2 minutes 13. Centrifuge at 6,000 g for 2 minutes to elute the purified DNA

DF Buffer, *W1 Buffer*, *Wash Buffer* and *Elution Buffer* are kit contents and their composition is not specified.

Protocol 7: Ligation (New England Biolabs® kit)

Mixing	1. In a PCR tube mix: -96 µl of ddH_2O -2 µl of *NE T4 Ligase Reaction Buffer* 10x -4 µl of DNA insert -4 µl of plasmid -1 µl of *NE T4 DNA Ligase*
Ligation	1. Leave at 4°C overnight

NE T4 Ligase Reaction Buffer: 50 mM Tris-HCl, 10 mM MgCl2, 10 mM Dithiothreitol, 1 mM ATP.

Protocol 8: Preparation of competent cells

Bacteria growth	1. Grow a 5 ml culture of Top10® *E.coli* overnight 2. Refresh the overnight culture (1 ml) in 100 ml of LB broth 3. Incubate the new culture at 37°C, 250rpm and check optical density (OD) at 595nm regularly until OD is greater than 0.4 4. Centrifuge during 10 minutes at 4000 g and 4°C and discard supernatant
Charging the bacteria	5. Add 50 ml of 0.1M $CaCl_2$ and resuspend the bacteria pellet 6. Centrifuge during 10 minutes at 4000 g and 4°C and discard supernatant 7. Add 40 ml of 0.1M $CaCl_2$, resuspend the pellet and leave on ice for 1 hour 8. Centrifuge during 10 minutes at 4000 g at 4°C and discard supernatant
Stocking	9. Add 3 ml of 0.1M $CaCl_2$ with 15% glycerol and resuspend the pellet 10. Aliquot 50µl per tube and store at -80°C

Protocol 9: Transformation

Mixing	1. Add 1µl of plasmid DNA into 50 µl of competent cells inside a microcentrifuge tube 2. Leave on ice during 30 minutes
Heat shock	3. Place the sample in a 42°C water-bath for 90 seconds 4. Chill on ice immediately after

Protocol 10: Colony selection by antibiotic resistance

Bacteria growth	1. Add 450µl of LB broth in the sample that just received the heat-shock 2. Incubate at 37°C, 250rpm for 1 hour
Antibiotic resistance selection	3. Centrifuge at 4000 g for 2 minutes and discard 400 µl of supernatant 4. Resuspend the pellet with the remaining supernatant 5. Streak 10 µl of the culture on a LB agar plate containing kanamycin (50 µg/ml) 6. Incubate at 37°C for 18 hours
Verification	7. Select colonies and analyze by plasmid digestion, PCR, or sequencing

LB Broth: 4 g NaCl + 2 g yeast extract + 4 g tryptone + 400 ml ddH_2O

Protocol 11: High-Speed Plasmid Mini Kit (Geneaid ®)

Harvesting	1. Transfer 1.5 ml of the cultured bacterial cells to a microcentrifuge tube 2. Centrifuge at 14,000 x g for 1minute and discard supernatant
Re-suspension	3. Add 200µl of *PD1 Buffer* (RNase) and resuspend the pellet
Lysis	4. Add 200µl of *PD2 Buffer* and mix gently (do not vortex)
Neutralization	5. Add 300µl of *PD3 Buffer* and mix gently (do not vortex) 6. Centrifuge at 14,000 x g for 3 minutes
DNA Binding	*7.* Place the *PD Column* in a 2 ml *Collection Tube* 8. Add the supernatant from step 6 to the *PD Column* and centrifuge at 14,000 x g for 30 seconds and discard the flow-through
Wash	9. Add 400 µl of *W1 Buffer* into the *PD Column* 10. Centrifuge at 14,000 x g for 30 seconds and discard the flow-through 11. Add 600 µl of *Wash Buffer* (ethanol added) into the *PD Column* 12. Centrifuge at 14,000 x g for 30 seconds and discard the flow-through 13. Centrifuge at 14,000 x g again for 3 minutes to dry the column matrix
DNA Elution	14. Transfer the dried *PD Column* to a new centrifuge tube and add 50 µl of *Elution Buffer* into the center of the column matrix and let stand 2 minutes 15. Centrifuge at 14,000 x g for 2 minutes to elute the DNA

PD1 Buffer, PD2 Buffer, PD3 Buffer, W1 Buffer, Wash Buffer and *Elution Buffer* are kit contents

Protocol 12: Small scale expression

Colony growing	1. Pick 1 isolated BL21 colony from the LB agar + kanamycin plate 2. Insert in a 5 ml LB broth + kanamycin culture medium 3. Incubate at 37°C and 250rpm overnight
Culture refresh	4. Transfer 350 µl of the culture in a new 7 ml LB broth + kanamycin culture medium and incubate at 37°C and 250rpm 5. After 2 hours of incubation, test the optical density of the medium using clean LB broth as standard. Repeat every 30 minutes until OD is greater than 0.6
Induction	6. Retrieve 1 ml of the culture medium, centrifuge at 4000 g during 5 minutes and store at -20°C (no induction control) 7. Add 0.8M IPTG at 1/1000 final concentration 8. Incubate at 37°C and 250rpm
Harvesting	9. After 3h, retrieve 1 ml of the culture medium, centrifuge at 4000 g during 5 minutes and store at -20°C (T_{3h} sample) 10. Repeat step 9 at 5h, 9h and 20h (T_{5h}, T_{9h} and T_{20h} samples)

Protocol 13: Large scale expression

Colony growing	1. Pick 1 isolated BL21 colony from the LB agar + kanamycin plate 2. Insert in a 10 ml LB broth + kanamycin culture medium 3. Incubate at 37°C and 250rpm overnight
Culture refresh	4. Pour the whole culture in 200 ml LB broth + kanamycin culture medium and incubate at 37°C and 250rpm 5. After 2 hours of incubation, test the optical density of the medium using clean LB broth as standard. Repeat every 30 minutes until OD is greater than 0.6
Induction	6. Retrieve 1 ml of the culture medium, centrifuge at 4000 g during 5 minutes and store at -20°C (no induction control) 7. Add 200 µl (1/1000) of 0.8M IPTG 8. Incubate at 25°C and 250rpm for 24 hours
Harvesting	9. Retrieve 1 ml of the culture as a "post-expression control" and centrifuge the rest of the culture at 4000 g during 15 minutes 10. Discard supernatant

Protocol 14: Protein extraction

Bacteria lysis	1. Resuspend the bacteria pellet obtained at the end of the large scale expression protocol with 10 ml of *binding buffer* (1/20 volume of refreshed bacteria) 2. Freeze (-80°C) and thaw (quick 37°C) 3. As soon as the frozen bacteria pellet is liquidized, add 200 µl of *lysozyme* (1/50 of buffer volume) and leave in a 37°C water-bath during 20 minutes 4. Do two more freeze-thaw cycles. Viscous should occur.
Sonication	5. Sonicate on ice to break down the chromosome DNA (work for 10s / pause for 10s, total action period: 5 minutes)
Harvesting of the native form	6. Centrifuge at 6,000 g at 4°C for 15 minutes 7. The supernatant contains the native protein, while the insoluble protein is trapped in the pellet. Retrieve the supernatant For pellet, see step 9 8. Add 200 µl (1/50) of *protease inhibitor*
Harvesting of the insoluble form	9. Resuspend the pellet from step 6 with 10 ml of *Urea binding buffer* and repeat steps 5 and 6 to solubilize the denatured protein

Lysozyme: 50 mg/ml

Binding buffer: 50 ml of 0.5M Tris-HCl + 50 ml of 5M NaCl + 0.34 g of Imidazole in 500 ml of ddH_2O.

Urea Binding buffer: 50 ml of 0.5M Tris-HCl + 1050 ml of 5M NaCl + 0.34 g of Imidazole + 240 g of urea in 500 ml of ddH_2O.

Protocol 15: His-tagged protein extraction (Chelating Sepharose Fast Flow of BioProcess™ Media family)

Column preparation	1. Transfer 1 ml of sepharose slurry to a new column and drip off the preserve medium 2. Use 5-time column volume of ddH_2O to wash the sepharose Discard flow-through 3. Charge the sepharose by adding 2-time volume of 0.1M NiSO4 Keep the column at room temperature for 5 minutes and drip off The sepharose should become green 4. Add 5-time column volume of ddH_2O and drip off 5. In order to equilibrate the column, add 5-time column volume of the same binding buffer that was used in extraction (*Binding Buffer* for native proteins and *Urea Binding Buffer* for denatured proteins Keep at room temperature for 5 minutes and drip off The column is ready for purification
His-tagged protein binding	6. Pour the supernatant obtained in the extraction protocol (save 100 µl as a control prior to purification) in the column 7. Rock for 2 hours at 4°C and drip off Collect the flow-through to later check binding efficiency (FT)
Discard the unbound proteins	8. Add 10-time column volume of *Washing Buffer 1* (*Urea Washing Buffer 1* for denatured proteins) into the column, rock for 5 minutes and drip off, keep the washing flow-through (W) 9. Check the protein content in the flow through by using *Bio-Red Protein Dye* reagent Repeat washes with *Washing Buffer 2 (Urea Washing Buffer* for denatured proteins) until the protein content in the flow-through is very low
Bound protein elution	10. Add 1 ml of *Elution Buffer 1* (*Urea Elution Buffer 1 for denatured proteins)* and rock for 5 minutes Collect flow-through in new tube (*E1*) and check protein content Repeat until protein content is very low (*E2*) 11. Repeat step 11 with *Elution Buffer 2 (Urea Elution Buffer 2)* until protein content is very low (*E3, E4, E5..)*
Regeneration	12. Add 1 ml of *Regenerating Buffer* into the column, rock for 5 minutes and collect flow-through (*R1)* Repeat (*R2)*

Bio-Red Protein Dye: 10 µl of flow through in 100 µl of 1x Bradford reagent

Binding Buffer: 50 ml of 0.5M Tris-HCl + 50 ml of 5M NaCl + 0.34 g of Imidazole) in 500 ml of ddH_2O

Urea Binding Buffer: 50 ml of 0.5M Tris-HCl + 100 ml of 5M NaCl + 0.34 g of Imidazole + 240 g of urea in 500 ml of ddH_2O

Washing buffer 1(20 mM): 50 ml of 0.5M Tris-HCl + 50 ml of 5M NaCl + 0.68 g of Imidazole in 500 ml of ddH2O

Urea Washing buffer 1(20 mM): 50 ml of 0.5M Tris-HCl + 100 ml of 5M NaCl + 0.68 g of Imidazole + 240 g of urea in 500 ml of ddH_2O

Washing buffer 2(50 mM): 50 ml of 0.5M Tris-HCl + 50 ml of 5M NaCl + 1.70 g of Imidazole in 500 ml of ddH2O

Urea Washing buffer2 (50 mM): 50 ml of 0.5M Tris-HCl + 100 ml of 5M NaCl + 1.70 g of Imidazole + 240 g of urea in 500 ml of ddH_2O

Elute buffer 1 (100 mM): 10 ml of 0.5M Tris-HCl + 10 ml of 5M NaCl + 0.68 g of Imidazole in 100 ml of ddH2O

Urea Elute buffer 2 (400 mM): 10 ml of 0.5M Tris-HCl + 20 ml of 5M NaCl + 2.72 g of Imidazole + 49 g of urea in 100 ml of ddH_2O

Regeneration buffer: 9.3 g of EDTA + 14.61 g of NaCl + 1.42 g of Na_2HPO_4 + 20 g of NaOH in 500 ml of ddH_2O – pH = 7.4

Protocol 16: SDS PAGE

Running gel preparation	1. Prepare the running gel (10 ml) by following the recipe shown in table 13 2. Pour the solution in the get casting form but leave 3cm for the stacking gel 3. Layer the top of the gel with 70% ethanol solution to remove the bubbles and avoid the top of the gel to dry 4. Wait 20 minutes for the gel to polymerize and discard the ethanol solution
Stacking gel preparation	5. Prepare the stacking gel (5 ml) by following the recipe shown in table 13 6. Pour the solution on top of the running gel 7. Insert combs and wait 20 minutes for the gel to polymerize
Sample preparation	8. Mix the sample 2:1 with the sample buffer 9. Heat the sample in a boiling water-bath for 5 minutes
Running the gel	10. Clamp in the gel and fill both buffer chambers with *Gel Running Buffer* 1. Pipet the samples into the wells and keep one well for the molecular weight standards 11. Run the gel at 80V for 25 minutes and then 120V for 70 minutes
Revelation	12. Retrieve the gel and stain it for 1 hour with *Brilliant Blue Staining Solution* 13. Destain several times with *Destaining Buffer* until the gel is clear

Running Buffer: 6.32 g Tris + 4 g Glycine + 1 g SDS + 1L ddH_2O

Brilliant Blue Staining Solution: 0.25 g Brilliant Blue R + 45 ml methanol + 45 ml ddH_2O + 10 ml acetic acid

Destaining Buffer: 300 ml methanol + 100 ml acetic acid + 600 ml ddH_2O

Table 13: Recipe for the running gel at 12.5% and the stacking gel at 4% used in SDS-PAGE

	Running gel 12.5% - pH8.8 (ml)	Stacking gel 4% - pH6.8 (ml)
ddH_2O	3.7	4.3
Acrylamid 40%	0.6	3
1.5M Tris	0.6	0
1M Tris	0	2.5
10% SDS	0.05	0.1
10% ammonium persulfate	0.05	0.1
TEMED	0.007	0.006

Protocol 17: Protein purification by transfer on PVDF membrane

Protein separation	1. Run each sample of eluate from the extraction protocol on a SDS-PAGE by adding 30 µl of sample and 15 µl of sample dye 3X in each well
Membrane transfer	2. Prepare the PVDF membrane: -soak the membrane 2 minutes in 100% ethanol -soak the membrane in ddH_2O for 3 minutes -soak the membrane in the *Blot Transfer Buffer* for 15 minutes 3. Transfer the gel to the PVDF membrane using the same technique as used for Western-blot (Western blot protocol can be found in the appendix)
MMP-9-Dist elution	4. Stain the membrane with Ponceau S and as soon as the band corresponding to MMP-9-Dist is visible, cut it from the membrane and destain it in ddH_2O 5. Insert the destained membrane piece in a microcentrifuge tube and add 300 µl of *Elution Buffer* 6. Vortex during 10 minutes, retrieve and discard the pieces of membrane

Elution Buffer: 2% SDS, 1% Triton-X in Tris-HCl 50 mM, pH 9.5.

Protocol 18: Acetone precipitation

Precipitation	1. Add cold (-20°C) *acetone* at a 4:1 volume ratio in the sample tube and store at -20°C for 1 hour 2. Centrifuge at 10000 g and 4°C for 15 minutes 3. Carefully discard the supernatant and add *washing solution* to clean the protein pellet 4. Centrifuge at 10000 g and 4°C for 10 minutes 5. Carefully discard supernatant 6. Air-dry for 2 minutes under the hood
Resuspension	7. Add PBS and resuspend the pellet until the protein is dissolved The volume of PBS is determined by the size of the pellet (80-400 µl)

Protocol 19: Western Blot

SDS-PAGE	1. Run the samples on a SDS-PAGE
Transfer preparation	2. Precut two Whatman™ 3MM filter papers and one nitrocellulose membrane (7x9cm) 3. Soak the membrane and filter paper in *Blot Transfer Buffer*
Assemble	4. In a try filled with *Blot Transfer Buffer*, assemble in the black side of a cassette in the following order: -black side of cassette -fiber pad -soaked filter paper -the gel from the SDS-PAGE -soaked nitrocellulose membrane -soaked filter paper -fiber pad -white side of cassette
Transfer	5. Insert the cassette into the electrode module, with the black side of the cassette next to the cathode 6. Place the electrode module in the buffer tank and fill the tank with *Blot Transfer Buffer* 7. Run electrophoresis at 250mA for 3 hours
Detection	8. Retrieve the membrane and block it in 10 ml of *5% skim milk* for 1 hour at room temperature 9. Add 1st antibody diluted in *5% skim milk* Incubate for 3 hours while shaking at room temperature 10. Wash 6 times for 5 minutes per wash with PBS-T 11. Add 2nd antibody diluted in *5% skim milk* and incubate 1 hour, shaking at room temperature 12. Wash 6 times for 5 minutes per wash with PBS-T
Revelation	13. Reveal the results by ECL (enhanced chemiluminescence) after addition of *VisGlow plus Chemiluminescent substrate*

Blot Transfer Buffer (1L): 800 ml ddH_2O + 200 ml methanol + 5.81 g tris + 2.93 g glycine
PBS-T (1L): 1L PBS + 1 ml Tween-20
5% skim milk (50 ml): 2.5 g Skim milk Difco™ + 50 ml PBS-T

Protocol 20: Ammonium sulfate precipitation

Retrieval of fraction 1 (0-40%)	1. Add an equal volume of ammonium sulfate until a 40% saturation is obtained Incubate 2 hours at room temperature 2. Centrifuge at 10,000 g for 10 minutes Retrieve supernatant and put it in a new tube for 50% precipitation 3. Resuspend the pellet in PBS (Fraction 1)
Retrieval of fraction 2 (40-50%)	4. Add ammonium sulfate in the retrieved supernatant until a 50% saturation is obtained Incubate 2 hours at room temperature
Verification	5. Run both fraction 1 and fraction 2 on a SDS-PAGE to verify which one contains the most antibody

Protocol 21: Immunoglobulin purification by Protein G (Protein G sepharose CL-4B – Pharmacia™)

Column Preparation	1. Transfer 2 ml of Protein G sepharose in a Biorad™ column 2. Wash column with 10 ml of *Wash Buffer 1*
Immunoglobulin Binding	3. Add the rabbit serum and rotate for 30 minutes at 4°C Run the column 4. Repeat step 3 with the flow-through
Wash	5. Wash the column with 10 ml of *Wash Buffer 1* 6. Wash the column with 10 ml of *Wash Buffer 2*
Antibody Elution	7. Elute the antibody off the Protein G beads with 2 ml of *Glycine Buffer and c*ollect the eluted protein in a tube containing a 15% final fraction volume of *Neutralization Buffer* (Fraction 1) 8. Repeat 3 times (Fraction 2, 3 and 4) 9. Wash the column with 10 ml of *Regeneration Buffer*
Verification	10. Check the samples on 10% SDS-PAGE, combine the peak fractions and test the antibody by western blotting

Wash Buffer 1: 0.135M NaCl, 100 mM Tris-HCl, pH8.0
Wash Buffer 2: 10 mM Tris-HCl, pH 8.0
Glycine Buffer: 100 mM glycine, pH3.0
Neutralization Buffer: 2M Tris-HCl, pH8.0

Protocol 22: Sandwich ELISA

Capture antibody coating	1. Dilute 13 µl of rabbit anti-NGAL antibody in 10.4 ml of *ELISA Coating Buffer* (1:800 dilution) 2. Fill all 96 wells of the ELISA plate with 100 µl of the solution 3. Close the plate, cover it with a wet tissue and put it in a plastic bag to avoid drying 4. Incubate at 37°C for 1 hour 5. Wash all wells 5 times with PBS
Blocking	6. Add 150 µl of *Blocking Buffer* in each well 7. Repeat step 3 and leave at room temperature for 1 hour 8. Empty the wells but do not wash
Add antigen	9. Insert *standard 1-8* and *standard PBS* in triplicates 10. Dilute 20 µl of each clinical sample into 380 µl of PBS and fill 3 wells (triplicate allow more reliable results) with 100 µl of this solution 11. Repeat step 3 and incubate at 4°C overnight 12. Wash all wells 5 times with PBS
Detecting antibody	13. Make a 1:3000 dilution of the anti-NGAL mouse antibody in *5% skim milk* 14. Apply 100 µl in each well 15. Repeat step 3 and incubate at 37°C for 1 hour 16. Wash all wells 5 times with PBS
Enzyme-linked antibody	17. Make a 1:5000 dilution of anti-mouse Fc section goat antibody in *5% skim milk* 18. Apply 100 µl in each well 19. Repeat step 3 and incubate at 37°C for 1 hour 20. Wash all wells 10 times with PBS
Substrate addition	21. Add 100 µl of *TMB* in each well 22. Close the plate and leave 5 minutes in darkness 23. Neutralize the reaction by adding 50 µl of 2M H_2SO_4 in each well
Revelation	24. Read the optical density of all the wells by spectrophotometry (wave length: 420-620nm)

ELISA Coating Buffer: 1.59 g of Na2CO3 + 2.93 g of NaHCO3 in 1L ddH_2O

Blocking Buffer: StartingBlock™ Blocking buffer

Protocol 23: IHC Protocol for NGAL and MMP-9

De-paraffinization and rehydration	1. Insert slides in 100% xylene solution at 50°C for 15 minutes two times 2. Dry the slides under the hood for 10 minutes 3. Soak the slides in 100% ethanol for 3 minutes two times 4. Soak the slides in 95% ethanol for 3 minutes 5. Soak the slides in 75% ethanol for 3 minutes 6. Soak the slides in 50% ethanol for 3 minutes
Antigen retrieval	7. Preheat *Citrate Buffer* with microwave until it boils 8. Soak the slides in the *Citrate Buffer* and microwave at 800 watts for 20 minutes 9. Wait 10 minutes for the *Citrate Buffer* to cool down using a room temperature water-bath
Blocking	10. Shake the slide dry and circle the area of interest with a *Dako pen* Be careful not to let the tissue dry out 11. Wash the slides 3 times with ddH_2O 12. Apply *3% H2O2* for 10 minutes 13. Wash the slides 3 times with ddH_2O 14. Apply *5% Goat serum* for 30 minutes 15. Wash the slides 3 times with ddH_2O
Primary antibody	16. Apply 300 µl of diluted primary antibody at the desired dilution (1:400 for NGAL, 1:150 for MMP-9) for 2 hours 17. Wash the slides 5 times with ddH_2O
Secundary antibody	18. Apply *Secondary antibody* for 30 minutes 19. Wash the slides 3 times with ddH_2O
Substrate	20. Apply 1:250 diluted *DAB* at the desired duration (2 minutes and 15 seconds for NGAL and 3 minutes for MMP-9) 21. Wash the slides 3 times with ddH_2O
Counterstaining	22. Apply Mayer's hematoxylin for 30 seconds 23. Wash the slides 3 times with ddH_2O 24. Soak the slides in tap water for 1 minute
Dehydration and mounting	25. Dry the slides at 50°C overnight 26. Mount the slides 27. Let the slides dry for 2 hours before reading

Citrate Buffer: 18 ml of 0.1M Citric acid + 82 ml of 0.1M Sodium citrate + 900 ml ddH_2O pH6.0

5% Goat serum: 0.5 ml of normal goat serum diluted in 9.5 ml of PBS

Primary antibody diluant: Dako REAL™ antibody diluant with background reducing components

Secundary antibody: Dako REAL™ EnVision™ HRP Rabbit/Mouse

DAB: Dako REAL™ DAB + Chromogen (x50) and Dako REAL™ Substrate buffer

APPENDIX 2: CANINE MMP-9 SEQUENCE

```
                                MMP9-1F-35 ►
                          G CAG CCC CTG GTC CTG GTG TTC
  1  ATG AGC CCC AGG CAG CCC CTG GTC CTG GTG TTC CTG GTG CTG GGC TGC TGC TCT GCA GCT CCC AGA CCA CAC AAG CCC ACC
     Met Ser Pro Arg Gln Pro Leu Val Leu Val Phe Leu Val Leu Gly Cys Cys Ser Ala Ala Pro Arg Pro His Lys Pro Thr

 82  GTT GTG GTC TTT CCA GGA GAC CTG AGA ACT AAT CTC ACT GAC AAG CAG CTG GCA GAG GAA TAT CTG TTT CGC TAT GGC TAC
     Val Val Val Phe Pro Gly Asp Leu Arg Thr Asn Leu Thr Asp Lys Gln Leu Ala Glu Glu Tyr Leu Phe Arg Tyr Gly Tyr

163  ACT CAA GTG GCC GAG CTG AGC GAC GAC AAG CAG TCC CTG AGT CGC GGG CTG CGG CTT CTC CAG AGG CGC CTG GCT CTG CCT
     Thr Gln Val Ala Glu Leu Ser Asp Asp Lys Gln Ser Leu Ser Arg Gly Leu Arg Leu Leu Gln Arg Arg Leu Ala Leu Pro

244  GAG ACT GGA GAG CTG GAC AAA ACC ACC CTG GAG GCC ATG CGG GCC CCG CGC TGC GGC GTC CCG GAC CTG GGC AAA TTC CAG
     Glu Thr Gly Glu Leu Asp Lys Thr Thr Leu Glu Ala Met Arg Ala Pro Arg Cys Gly Val Pro Asp Leu Gly Lys Phe Gln

325  ACC TTT GAG GGC GAC CTC AAG TGG CAC CAC AAC GAC ATC ACT TAC TGG ATA CAA AAC TAC TCG GAA GAC TTG CCC CGC GAC
     Thr Phe Glu Gly Asp Leu Lys Trp His His Asn Asp Ile Thr Tyr Trp Ile Gln Asn Tyr Ser Glu Asp Leu Pro Arg Asp

406  GTG ATC GAC GAC GCC TTT GCC CGA GCC TTC GCG GTC TGG AGC GCG GTG ACA CCG CTC ACC TTC ACT CGC GTG TAC GGC CCC
     Val Ile Asp Asp Ala Phe Ala Arg Ala Phe Ala Val Trp Ser Ala Val Thr Pro Leu Thr Phe Thr Arg Val Tyr Gly Pro

                          ◄ MMP9-1R-544
                G TAG TAG TAA GTC AAA CCA CAA
487  GAA GCC GAC ATC ATC ATT CAG TTT GGT GTT AGG GAG CAC GGA GAT GGG TAT CCC TTC GAT GGG AAG AAC GGG CTT CTG GCT
     Glu Ala Asp Ile Ile Ile Gln Phe Gly Val Arg Glu His Gly Asp Gly Tyr Pro Phe Asp Gly Lys Asn Gly Leu Leu Ala

568  CAC GCC TTT CCT CCC GGC CCG GGC ATT CAG GGA GAC GCC CAC TTC GAC GAC GAG GAG TTA TGG ACT CTG GGC AAG GGC GTC
     His Ala Phe Pro Pro Gly Pro Gly Ile Gln Gly Asp Ala His Phe Asp Asp Glu Glu Leu Trp Thr Leu Gly Lys Gly Val

649  GTG GTT CCG ACC CAC TTC GGA AAC GCA GAT GGC GCC CCC TGC CAC TTC CCC TTC ACC TTC GAG GGC CGC TCC TAC TCG GCC
     Val Val Pro Thr His Phe Gly Asn Ala Asp Gly Ala Pro Cys His Phe Pro Phe Thr Phe Glu Gly Arg Ser Tyr Ser Ala

730  TGC ACC ACG GAC GGC CGC TCC GAT GAC ACG CCC TGG TGC AGC ACC ACG GCC GAC TAT GAC ACC GAC CGT CGG TTC GGC TTC
     Cys Thr Thr Asp Gly Arg Ser Asp Asp Thr Pro Trp Cys Ser Thr Thr Ala Asp Tyr Asp Thr Asp Arg Arg Phe Gly Phe

811  TGC CCC AGC GAG AAA CTC TAC ACC CAG GAC GGC AAT GGG GAC GGC AAG CCC TGC GTG TTT CCG TTC ACC TTC GAG GGC CGC
     Cys Pro Ser Glu Lys Leu Tyr Thr Gln Asp Gly Asn Gly Asp Gly Lys Pro Cys Val Phe Pro Phe Thr Phe Glu Gly Arg

892  TCC TAC TCC ACG TGC ACC ACC GAC GGC CGC TCG GAC GGC TAC CGC TGG TGC TCC ACC ACC GGC GAC TAC GAC CAG GAC AAA
     Ser Tyr Ser Thr Cys Thr Thr Asp Gly Arg Ser Asp Gly Tyr Arg Trp Cys Ser Thr Thr Gly Asp Tyr Asp Gln Asp Lys

973  CTC TAC GGC TTC TGC CCA ACC CGA GTC GAT TCC GCG GTG ACC GGG GGC AAC TCC GCC GGG GAG CCG TGT GTC TTC CCC TTC
     Leu Tyr Gly Phe Cys Pro Thr Arg Val Asp Ser Ala Val Thr Gly Gly Asn Ser Ala Gly Glu Pro Cys Val Phe Pro Phe

1054  ATC TTC CTG GGC AAG CAG TAC TCG ACG TGC ACC AGG GAG GGC CGC GGA GAT GGG CAC CTC TGG TGC GCC ACC ACT TCG AAC
      Ile Phe Leu Gly Lys Gln Tyr Ser Thr Cys Thr Arg Glu Gly Arg Gly Asp Gly His Leu Trp Cys Ala Thr Thr Ser Asn

1135  TTT GAC AGA GAC AAG AAG TGG GGC TTC TGC CCG GAC CAA GGA TAC AGC CTG TTC CTT GTG GCC GCC CAT GAG TTC GGC CAC
      Phe Asp Arg Asp Lys Lys Trp Gly Phe Cys Pro Asp Gln Gly Tyr Ser Leu Phe Leu Val Ala Ala His Glu Phe Gly His

1216  GCG CTG GGT TTA GAT CAT TCA TCG GTG CCA GAA GCG CTC ATG TAC CCC ATG TAC AGC TTC ACC GAG GGC CCC CCC CTG CAT
      Ala Leu Gly Leu Asp His Ser Ser Val Pro Glu Ala Leu Met Tyr Pro Met Tyr Ser Phe Thr Glu Gly Pro Pro Leu His

1297  GAA GAC GAC GTG AGG GGC ATC CAG CAT CTG TAC GGT CCT CGC CCT GAA CCT GAG CCA CAG CCT CCA ACC GCC CCG CCC ACC
      Glu Asp Asp Val Arg Gly Ile Gln His Leu Tyr Gly Pro Arg Pro Glu Pro Glu Pro Gln Pro Pro Thr Ala Pro Pro Thr

1378  GCC CCG CCC ACC GTC TGC GCT ACT GGT CCT CCC ACC ACC CGC CCC TCA GAG CGC CCC ACT GCT GGC CCC ACA GGC CCC CCT
      Ala Pro Pro Thr Val Cys Ala Thr Gly Pro Pro Thr Thr Arg Pro Ser Glu Arg Pro Thr Ala Gly Pro Thr Gly Pro Pro

1459  GCA GCT GGC CCC ACG GGT CCC CCC ACT GCT GGC CCC TCT GAG GCC CCT ACA GTG CCT GTG GAT CCG GCA GAG GAT ATA TGC
      Ala Ala Gly Pro Thr Gly Pro Pro Thr Ala Gly Pro Ser Glu Ala Pro Thr Val Pro Val Asp Pro Ala Glu Asp Ile Cys

                                MMP9-1F-1580 ►
                          GCC ATC GCG GAG ATC AGG AAC TAC
1540  AAA GTG AAC ATC TTC GAC GCC ATC GCG GAG ATC AGG AAC TAC TTG CAT TTC TTC AAG GAA GGG AAG TAC TGG CGA TTC TCC
      Lys Val Asn Ile Phe Asp Ala Ile Ala Glu Ile Arg Asn Tyr Leu His Phe Phe Lys Glu Gly Lys Tyr Trp Arg Phe Ser

1621  AAG GGC AAG GGA CGC CGG GTG CAG GGC CCC TTC CTT ATC ACC GAC ACG TGG CCT GCG CTG CCC CGC AAG CTG GAC TCC GCC
      Lys Gly Lys Gly Arg Arg Val Gln Gly Pro Phe Leu Ile Thr Asp Thr Trp Pro Ala Leu Pro Arg Lys Leu Asp Ser Ala

1702  TTT GAG GAC GGG CTC ACC AAG AAG ACT TTC TTC TTC TCT GGG CGC CAA GTG TGG GTG TAC ACA GGC ACG TCG GTG GTA GGC
      Phe Glu Asp Gly Leu Thr Lys Lys Thr Phe Phe Phe Ser Gly Arg Gln Val Trp Val Tyr Thr Gly Thr Ser Val Val Gly

1783  CCG AGG CGT CTG GAC AAG CTG GGC CTG GGC CCG GAG GTT ACC CAA GTC ACC GGC GCC CTC CCG CAA GGC GGG GGT AAG GTG
      Pro Arg Arg Leu Asp Lys Leu Gly Leu Gly Pro Glu Val Thr Gln Val Thr Gly Ala Leu Pro Gln Gly Gly Gly Lys Val

1864  CTG CTG TTC AGC AGG CAG CGC TTC TGG AGT TTC GAC GTG AAG ACG CAG ACC GTG GAT CCC AGG AGC GCC GGC TCG GTG GAA
      Leu Leu Phe Ser Arg Gln Arg Phe Trp Ser Phe Asp Val Lys Thr Gln Thr Val Asp Pro Arg Ser Ala Gly Ser Val Glu

1945  CAG ATG TAC CCC GGG GTG CCC TTG AAC ACG CAT GAC ATC TTC CAG TAC CAA GAG AAA GCC TAC TTC TGC CAG GAC CGC TTC
      Gln Met Tyr Pro Gly Val Pro Leu Asn Thr His Asp Ile Phe Gln Tyr Gln Glu Lys Ala Tyr Phe Cys Gln Asp Arg Phe

                                                                  ◄ MMP9-1R-2131
                                                        G AAA CTG TAA AAC GTC ACG G
2026  TAC TGG CGT GTG AAT TCT CGG AAT GAG GTG AAC CAG GTG GAC GAA GTG GGC TAC GTG ACC TTT GAC ATT TTG CAG TGC CCT
      Tyr Trp Arg Val Asn Ser Arg Asn Glu Val Asn Gln Val Asp Glu Val Gly Tyr Val Thr Phe Asp Ile Leu Gln Cys Pro

2107  GAG GAT TAG
      Glu Asp ---
```

Figure 28: Canine MMP-9 Gene sequence

The translated amino-acids are marked as well as the location of the primers that were chosen

APPENDIX 3: PET-24A(+) MAP

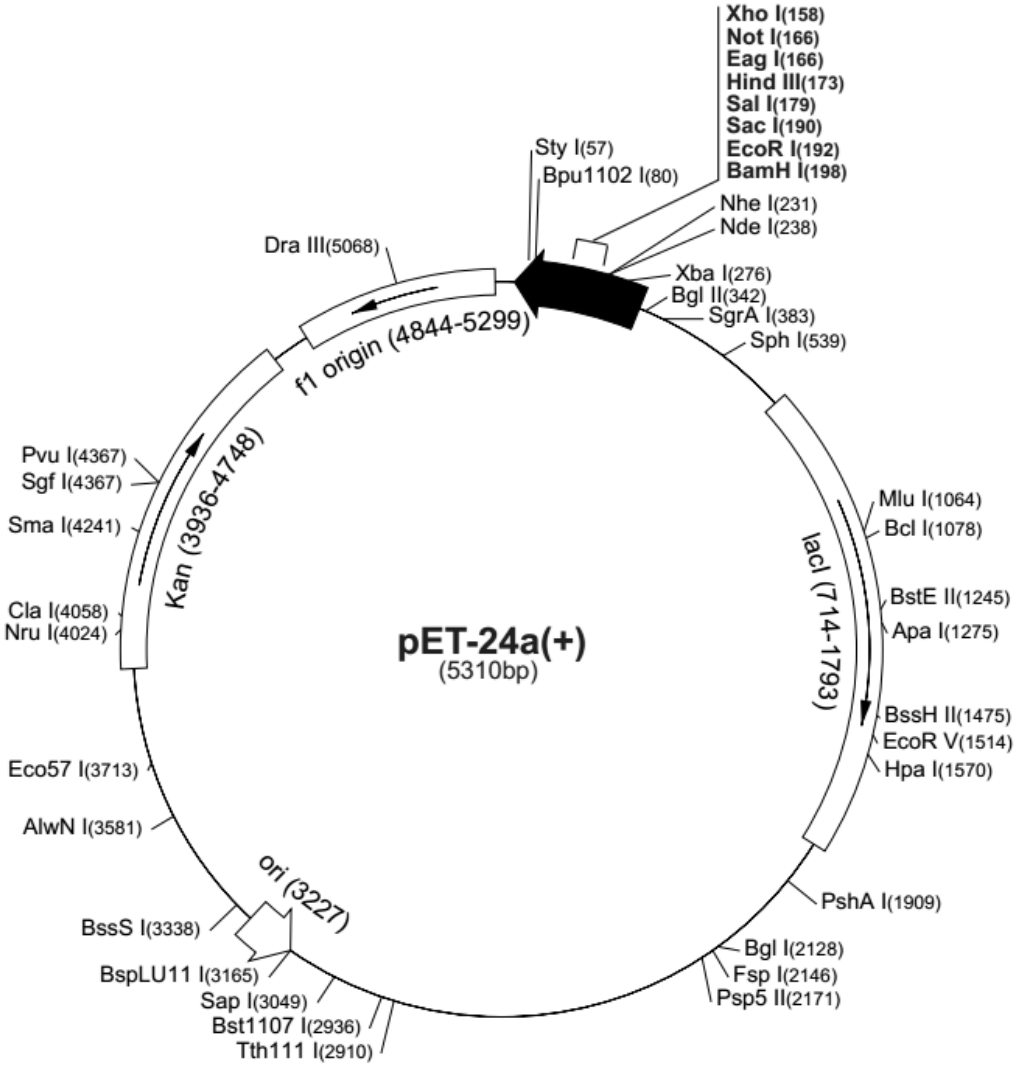


Figure 29: Map of pET-24a(+) vector

The kanamycin resistance gene as well as the unique restriction sites are marked. The black bold arrow marks the expression region detailed in figure 8.

APPENDIX 4: ANTIGEN CONCENTRATION ESTIMATION

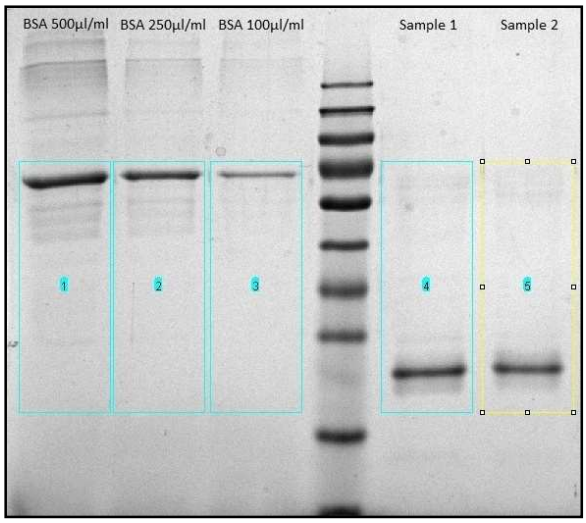


Figure 30: Concentration estimation of the antigen by SDS-PAGE

The standards used are 3 different concentration of BSA (100, 250 and 500 µl/ml). *Image J* software can calculate the intensity of the signal after circling the area of interest. Note that the protein in samples is very pure and very little contaminants can be noticed.

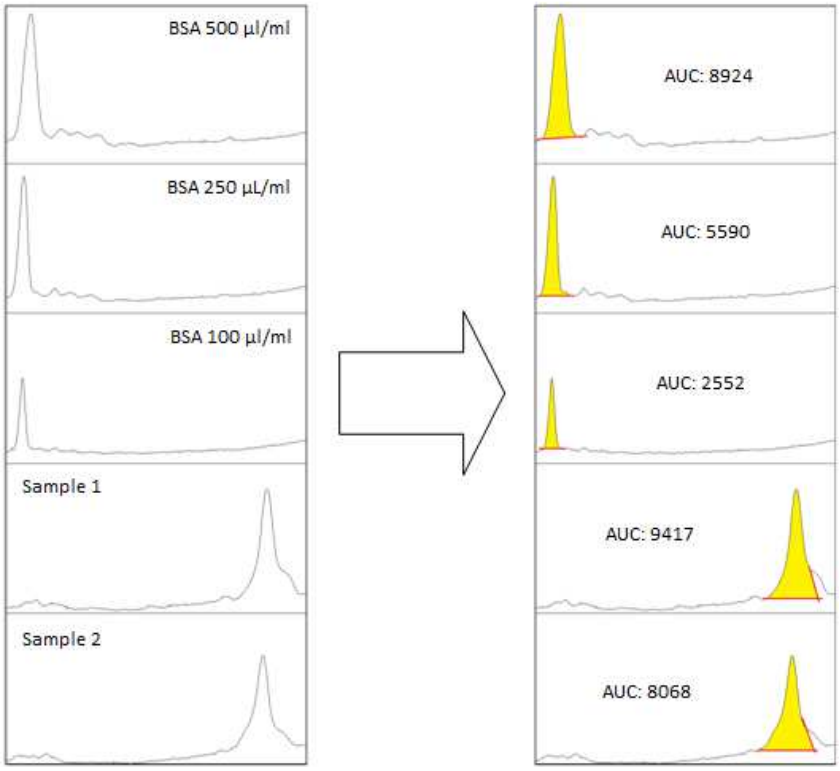


Figure 31: Determination of the antigen's signal intensity compared to the BSA standards

Image J software draws the color intensity curve. The area corresponding to the BSA and the antigen are then determined and the software calculates their AUC, which is proportional to the concentration.

APPENDIX 5: MICE IMMUNIZATION SCHEME

Table 14: Immunization scheme of the three mice with MMP-9-Dist

Mouse 1

	Priming	1st Boost	2nd Boost	3rd Boost
Day	1	11	22	31
Concentration	143 µg/ml	390 µg/ml	760 µg/ml	240 µg/ml
Volume	300 µl	140 µl	140 µl	300 µl
Antigen mass	43 µg	54.6 µg	106 µg	80 µg
Purity	99%	99%	99%	99%

Mouse 2

	Priming	1st Boost	2nd Boost	3rd Boost
Day	2	11	22	31
Concentration	361 µg/ml	154 µg/ml	680 µg/ml	410 µg/ml
Volume	250 µl	300 µl	140 µl	200 µl
Antigen mass	90 µg	46 µg	95 µg	82 µg
Purity	70%	70%	99%	99%

Mouse 3

	Priming	1st Boost	2nd Boost	3rd Boost
Day	2	11	22	31
Concentration	602 µg/ml	262 µg/ml	810 µg/ml	370 µg/ml
Volume	150 µl	300 µl	120 µl	220 µl
Antigen mass	90 µg	78 µg	97 µg	81 µg
Purity	70%	70%	99%	99%

FRENCH SUMMARY OF THE STUDY

INTRODUCTION

Les tumeurs de la glande mammaire représentent le type de tumeur le plus fréquemment rencontré chez les chiennes, avec une incidence affectant plus de 25% des femelles non stérilisées. Cette incidence est plus de trois fois supérieure à celle du cancer du sein observée en médecine humaine. De nouvelles études sont constamment effectuées afin de trouver de nouvelles façons de diagnostiquer, d'évaluer, de suivre et de traiter cette pathologie chez ces deux espèces.

La matrix metalloprotéinase 9 (MMP-9), aussi décrite sous le nom de gelatinase B, est un contributeur majeur dans la dégradation protéolytique de la matrice extra-cellulaire lors de l'invasion des tissus adjacents par une tumeur. En médecine humaine, il a été montré que l'expression de cette protéine dans le sérum, l'urine et le tissu mammaire de patientes atteintes de cancer du sein était fortement augmentée. De nombreux auteurs ont donc suggéré l'utilisation de la MMP-9 comme bio-marqueur de cette pathologie.

La neutrophil gelatinase-associated lipocalin (NGAL) a été isolée pour la première fois en 1992 en tant que glycoprotéine de petite taille, fixée de manière covalente à la MMP-9 dans des granulocytes neutrophiles humains. Il fut montré plus tard que la NGAL pouvait inhiber la dégradation de la gelatinase B, augmentant ainsi l'activité de cette dernière. Il fut par ailleurs démontré que cette protéine aussi pouvait être utilisée comme bio-marqueur du cancer du sein dans le sérum, l'urine et le tissu mammaire.

En médecine vétérinaire, la corrélation entre l'expression de ces deux protéines et les tumeurs mammaires n'a que très peu été étudiée. Seuls quelques projets rapportent l'utilisation d'anticorps anti MMP-9 humaine dans des études immuno-histochimiques et démontrent que son expression était significativement corrélée à plusieurs facteurs pronostiques tels que la malignité, le grade histologique ou la présence de métastases dans les nœuds lymphatiques. En revanche, aucune étude n'a été menée sur la NGAL canine.

Cette étude avait pour but d'utiliser des anticorps spécifiques de la NGAL et de la MMP-9 canines afin de mettre au point une méthode permettant d'évaluer le niveau d'expression de ces deux protéines dans plusieurs échantillons biologiques de patients atteints de tumeur mammaire. L'expression sérique et urinaire de la NGAL a été analysée grâce à un test ELISA fait maison, tandis que les niveaux d'expression de la NGAL et de la MMP-9 dans des coupes histologiques de tumeurs mammaires ont été évalués par immuno-histochimie.

1. MATERIEL ET METHODE

1.1. Production d'anticorps anti MMP-9 canine

- Extraction de l'ARN total et transcription en ADN

L'ARN total de cellules issues de tissu testiculaire canin a été isolé après lyse à l'azote liquide et extraction à l'aide d'un kit (*Qiagen®'s RNeasy®*). L'ARN total a ensuite été transcrit en ADNc par transcription inverse grâce au kit *Superscript™ III Supermix* de *Invitrogen™*.

- Production d'un insert codant pour la MMP-9-Dist

Après analyse de l'hydrophobicité de la MMP-9 canine, il a été décidé de produire comme antigène la partie distale de la protéine (nommée MMP-9-Dist dans cette étude) correspondant environ aux 200 derniers acides aminés. La fraction d'ADN codant pour cette partie distale a été amplifiée par PCR en utilisant les amorces suivantes :

5'-AACATATGGCCATCGCGGAGATCAGGAACTAC-3'
et 5'-AACTCGAGGCACTGCAAAATGTCAAAG-3'.

Le produit obtenu a été vérifié par électrophorèse sur gel d'agarose à 1% et présentait une taille compatible avec celle de la portion d'ADN désirée, soit environ 550pdb.

- Préparation du vecteur

Le vecteur utilisé était le plasmide pET-24a de *Novagen™*. pET-24a possède un gène de résistance à la kanamycine et permet notamment d'ajouter une étiquette de six résidus d'histidine à une extrémité de la protéine. Le plasmide a d'abord été digéré par deux enzymes de restriction (NdeI et XhoI) et le résultat de cette digestion a été vérifié par électrophorèse sur gel d'agarose à 1%. L'insert codant pour la MMP-9-Dist a lui aussi été digéré à l'aide des mêmes enzymes et du même protocole.

- Liaison et transformation

La liaison entre l'insert et le vecteur a été obtenue à l'aide de l'ADN ligase en incubant le mélange une nuit à 4°C. Le plasmide ainsi généré a été inséré dans des *Escherichia coli* One Shot® Top10 d'Invitrogen®. Cette transformation a été réalisée en mélangeant les bactéries préalablement chargées positivement par du $CaCl_2$, avec le plasmide et en leur faisant subir un choc thermique à 42°C pendant 30 secondes afin de forcer l'internalisation du plasmide dans la bactérie.

- Sélection et screening des colonies bactériennes

Une première sélection a été réalisée en utilisant comme milieu de culture un milieu LB enrichi en kanamycine, afin de ne sélectionner que les bactéries ayant acquis un gène de résistance et donc le plasmide. Un premier screening a été appliqué en amplifiant par PCR, à partir de bactéries isolées re-suspendues, la portion d'ADN codant pour la MMP-9-Dist. Un second test de screening a été réalisé en extrayant les plasmides des bactéries à l'aide du kit *High Speed Plasmid Mini Kit* de Geneaid®, et en étudiant les produits obtenus après digestion par les enzymes NdeI et XhoI. Les produits de ces deux tests de screening ont été vérifiés par électrophorèse sur gel d'agarose à 1% et coïncidaient avec les résultats attendus. Afin de confirmer que les bactéries avaient efficacement intégré la portion d'ADN codant pour la MMP-9-Dist, un échantillon a été envoyé pour séquençage au laboratoire Mission Biotech de Taipei. La séquence extraite était identique à celle de MMP-9-Dist.

- Transfert du plasmide chez un hôte expresseur

Les *E.coli* top10 ne pouvant que répliquer le plasmide et non exprimer l'insert s'y trouvant, les plasmides ont été extrait à l'aide du kit de Geneaid®, et ont été réinsérés chez un nouvel hôte (*E.coli* BL21 de Biomol®) en répétant à l'identique le protocole utilisé pour la première transformation. Les bactéries ayant efficacement intégré le plasmide ont ensuite été sélectionnées en utilisant un milieu de culture enrichi en kanamycine.

- Expression de l'antigène

Après avoir rafraichi une culture à un ratio de 1/20 dans un grand volume de milieu LB (200-500 ml), celle-ci a été incubée à 37°C jusqu'à ce que sa densité optique à 595nm soit supérieure à 0.6A. L'expression a ensuite été induite par de l'IPTG à 0.8 mM et a été réalisée à 25°C pendant 24 heures.

- Extraction de la MMP-9-Dist recombinante

Les bactéries ont été lysées à l'aide de lysozyme (30 minutes à 37°C) et de cycles de chocs thermiques (-80°C et +37°C) avant de subir 10 minutes de sonication aux ultrasons puis une centrifugation. La MMP-9-Dist recombinante étant en grande majorité exprimée sous sa forme dénaturée, il a fallu répéter les étapes de sonication et de centrifugation en utilisant un tampon riche en urée (8M) et en NaCl (1M) afin de rendre la forme dénaturée soluble.

La protéine a ensuite été extraite à l'aide de colonnes de chromatographie contenant *GE Healthcare's Chelating Sepharose*™ qui, une fois chargées par du Ni^{2+}, montrent une très forte affinité pour les résidus histidine et donc en particulier pour l'étiquette histidine présente sur la MMP-9-Dist recombinante. L'imidazole permettant de réduire cette affinité, des premiers nettoyages avec des concentrations de 20 mM et 50 mM d'imidazole ont été réalisés

afin de se débarrasser des contaminants. La MMP-9-Dist a ensuite été éluée en augmentant la teneur en imidazole à 400 mM.

- Purification de la MMP-9-Dist recombinante

Les protéines présentes dans les éluats de l'étape d'extraction ont été séparées par SDS-PAGE et transférées sur une membrane PVDF puis teintes avec du Ponceau S. Dès que la MMP-9-Dist fut visible, elle a été repérée, découpée et le fragment de membrane inséré dans un microtube contenant une solution tampon d'élution. Après 10 minutes de vortex, le fragment de membrane a été enlevé et l'échantillon a subi une précipitation à l'acétone afin de laver la protéine des contaminants de la solution d'élution. L'échantillon a donc été mélangé à de l'acétone froid (-20°) et a été maintenu à -20°C pendant 1 heure afin que la MMP-9-Dist précipite. Après centrifugation, l'acétone a été retirée et le culot a été re-suspendu dans du PBS.

- Estimation de la concentration de l'antigène

L'échantillon obtenu ainsi que différentes concentrations de BSA (bovine serum albumin) ont été séparés par SDS-PAGE. L'intensité du signal obtenu pour MMP-9-Dist a pu être comparée à celles des standards de BSA afin d'évaluer, à l'aide du logiciel *Image J*, la concentration en MMP-9-Dist dans l'échantillon.

- Immunisation et retrait des antigènes

Après vérification par Western-Blot que la protéine était bien la MMP-9-Dist recombinante (par mise en évidence de la présence d'une étiquette d'histidines), l'antigène a été mélangé avec du *Freund's adjuvant complete sigma* (ratio 1:1) puis injecté par voie intrapéritonéale à 3 souris. Une primo-injection et trois rappels ont été réalisés. Avant chaque injection, 500 µl de sang étaient prélevés depuis le sinus rétro-orbital. Un dernier échantillon de sang a été prélevé une semaine après le dernier rappel puis centrifugé et le sérum obtenu a été utilisé comme anticorps polyclonal de la MMP-9 canine.

1.2. Production et purification d'anticorps anti NGAL canine

- Production de NGAL recombinante

La production de la NGAL recombinante n'a pas été réalisée lors de ce projet mais lors d'une étude préliminaire réalisée au sein du Graduate Institute of Microbiology and Public Health de Chung Hsing University (Taichung, Taiwan). L'ARN total a été extrait d'un homogénat de tissu mammaire canin et a été transcrit en ADNc. La portion codante pour la NGAL a été amplifiée par PCR, insérée dans un vecteur pET-32b puis intégrée dans des *E.coli* BL21. La

NGAL recombinante a été exprimée à 16°C pendant 24 heures après induction par de l'IPTG, puis extraite par chromatographie sur des colonnes contenant du sepharose chargé au nickel. Trois souris et trois lapins ont été immunisés.

- Purification des anticorps anti-NGAL

Lors de cette étude, le sérum des lapins et des souris a été récupéré et afin de purifier et d'enrichir l'anticorps en vue de l'IHC (Immunohistochimie), deux méthodes de purification ont été utilisées. Dans un premier temps, une précipitation au sulfate d'ammonium a permis de ne conserver que la fraction de protéines qui précipitait à une saturation en sulfate d'ammonium inférieure à 50%. Les anticorps ont ensuite été purifiés par chromatographie à l'aide de protéines G qui se lient avec une forte affinité au fragment Fc des anticorps. Une solution de glycine à pH 3.0 a permis ensuite de défaire cette liaison et de récupérer les anticorps après avoir éliminé les contaminants.

1.3. Mise au point d'un test sandwich ELISA pour doser la NGAL

- Echantillons étudiés

En vue d'étudier le niveau d'expression de la NGAL dans le sérum et dans l'urine de chiennes malades, 28 échantillons de sang et 8 échantillons d'urine de chiennes atteintes de tumeur mammaire maligne ont été analysés dans cette étude. Les témoins, provenant de chiennes saines, étaient au nombre de 14 pour les échantillons sanguins et de 5 pour ceux d'urine.

- Optimisation du protocole

L'anticorps anti-NGAL provenant du sérum d'un lapin a été utilisé comme anticorps de capture à un taux de dilution de 1:800. Les 96 puits de la plaquette ELISA ont été remplis avec 100 µl et incubés 1h à 37°C. Entre chacune des étapes suivantes, les puits ont été rincés cinq fois avec du PBS-T.

150 µl de solution tampon pour blocage ont ensuite été ajoutés dans chaque puits et l'ensemble a été laissé 1h à température ambiante.

20 µl de chaque échantillon clinique ont ensuite été dilués dans 380 µl de PBS et pour chaque échantillon, trois puits ont été remplis avec 100 µl du mélange obtenu, puis la plaque ELISA a été incubée à 4°C pendant une nuit.

L'anticorps anti-NGAL provenant du sérum d'une souris a ensuite servi d'anticorps de détection. 100 µl d'anticorps dilué à 1:3000 ont été utilisés avant d'incuber à 37°C pendant 1h.

Un anticorps anti-Fc-souris, conjugué à une peroxydase et dilué à 1:5000 dans 100 µl ont ensuite été ajoutés. Après 1h d'incubation à 37°C et de nombreux lavages au PBS-T, 100 µl de TMB ont été ajoutés dans chaque puits et laissés pendant 5 minutes avant que la réaction ne soit stoppée par un ajout de 50 µl de H_2SO_4. La densité optique des 96 puits a ensuite été mesurée (420-620nm).

1.4. Mise au point d'un protocole d'immunohistochimie en vue de doser la NGAL et la MMP-9 dans le tissu mammaire

- Nature et origine des échantillons

43 échantillons ont été obtenus lors d'exérèses chirurgicales réalisées à l'hôpital vétérinaire de l'Université de Chung Hsing sur des chiennes atteintes de tumeur mammaire. 32 des tumeurs étudiées étaient malignes (20 carcinomes complexes, 11 carcinomes simples, 1 carcinosarcome) et 11 étaient bénignes (8 tumeurs bénignes mixtes, 2 adénomes complexes, 1 adénome simple). Nos essais ont été réalisés sur des sections de 4µm de ces tissus ainsi que sur 13 sections de glandes mammaires saines.

- Optimisation du protocole d'IHC

Les lames ont d'abord été déparaffinées à l'aide de deux bains successifs de 15 minutes dans du xylène à 50°C puis réhydratées progressivement par bains de 3 minutes dans des solutions d'éthanol de moins en moins concentrées (100% deux fois de suite, 95%, 75% puis 50%).

Les antigènes ont ensuite été rendus détectables en chauffant les lames dans une solution tampon citratée pendant 20 minutes à 800 watts au four à micro-ondes. Après refroidissement à température ambiante, la section de tissu a été entourée avec un feutre *Dakopen*. Entre chacune des étapes suivantes, les lames ont été rincées avec de l'eau distillée.

Afin de minimiser le marquage non spécifique, deux méthodes de blocage ont été utilisées : tout d'abord les peroxydases endogènes ont été inhibées par une solution à 3% de H_2O_2 pendant 10 minutes, puis une solution de sérum de chèvre à 5% a été appliquée sur les lames pendant 30 minutes. Le premier anticorps a ensuite été ajouté, dilué à 1:400 pour la NGAL et 1:100 pour la MMP-9, et laissé en contact avec le tissu pendant 2 heures.

L'anticorps dilué *Dako REAL™ EnVision™*, couplé à une peroxydase a ensuite été ajouté puis laissé 30 minutes avant l'ajout de DAB (diaminobenzidine) dilué à 1:250. Le temps de mise en contact du tissu et de ce substrat était de 2 minutes 15 secondes pour la NGAL et de 3 minutes pour la MMP-9.

Une contre coloration à l'hématoxyline de 30 secondes a été réalisée avant de plonger les lames dans de l'eau plate pendant 1 minute. Les lames ont ensuite été séchées une nuit à 50°C puis montées.

- Contrôles positifs et négatifs

Contrôle positif : Pour chaque nouvelle série, une lame que l'on savait être fortement positive était incluse. S'il était jugé que la qualité du marquage obtenu sur cette lame était moins bonne que d'habitude, toutes les lames de la série étaient écartées et l'opération répétée.

Contrôle négatif : Chaque nouvelle série contenait une lame de contrôle négatif pour laquelle l'anticorps primaire était remplacé par du sérum pré-immun.

- Lecture des lames et score de marquage

Pour chaque lame, les paramètres suivants étaient analysés : existence et spécificité du marquage, distribution cellulaire, homogénéité à travers la section. De plus, l'expression des protéines était évaluée de manière semi-quantitative en jugeant :

-le score d'intensité, allant de 0 (pas de marquage) à 3 (équivalent au contrôle positif).
-le score de proportion de cellules tumorales positives, pouvant être 1 (<10%), 2 (11-50%), 3 (50-80%) ou 4 (>80%).

Le score final (FS) d'une lame était ensuite égal au produit de ces deux scores. L'échantillon était ainsi considéré négatif (FS entre 0 et 2), faiblement positif (FS entre 3 et 7) ou fortement positif (FS entre 7 et 12). Les lames ont été scorées par deux évaluateurs. Lorsque les avis étaient différents, le score final était discuté jusqu'à obtention d'un accord.

1.5. Analyses statistiques

Les tests statistiques utilisés dans cette étude sont le test de Student (avec ou sans la correction d'Aspin-Welch selon l'homogénéité des variances), le test exact de Fisher et le test de corrélation de Spearman. Tous ont été réalisés à l'aide du logiciel GraphPad™ Prism et tout résultat associé à une valeur $p<0.05$ était considéré comme significatif.

2. RESULTATS

2.1. Production d'anticorps anti MMP-9 canine

- Capacité à détecter la MMP-9

Après retrait du sérum des souris immunisées avec la MMP-9-Dist, son aptitude à détecter la MMP-9 a été testée par Western blot sur trois échantillons :

-la MMP-9-Dist recombinante, afin de s'assurer que l'anticorps reconnaissait l'antigène initial

-la MMP-9 humaine du commerce, afin de tester la capacité de l'anticorps à reconnaitre la protéine humaine

-un homogénat tissulaire de carcinome mammaire, afin de voir si l'anticorps était apte à détecter la protéine sur des échantillons cliniques.

Pour chacun de ces trois essais, l'anticorps a su reconnaître la protéine.

- Evaluation du titre en anticorps du sérum

Un ELISA a été réalisé en utilisant la MMP-9-Dist recombinante comme antigène et en variant le taux de dilution (de 1:100 à 1:3200) des sérums obtenus chez les 3 souris soumises à l'immunisation. Le titre était défini comme étant la concentration en anticorps pour laquelle la densité optique obtenue était deux fois supérieure à celle du sérum pré-immun. Le sérum utilisé pour les essais d'IHC présentait ainsi un titre de 1:5840.

2.2. Evaluation du niveau d'expression de la NGAL dans le sérum et l'urine par ELISA sandwich

- Echantillons de sang

La moyenne de la densité optique observée chez les chiennes atteintes de tumeur mammaire maligne (0.147, n=28) était supérieure à celle observée chez les témoins sains (0.128, n=14) mais cette différence n'était pas statistiquement significative (test de Student avec correction d'Aspin-Welsh, p=0.116).

Cependant, l'implémentation d'une valeur seuil à une densité optique de 0.193 (égale à la moyenne observée chez les individus sains + 2 écarts-types), montre que les échantillons de chiennes malades dépassent plus souvent ce seuil que les échantillons témoins (Test de Fisher, p=0.015).

- Echantillons d'urine

La moyenne de la densité optique observée pour les échantillons d'urine de chiennes malades (0.097, n=8) était supérieure à celle des témoins sains (0.048, n=5). La différence était statistiquement significative (test de Student avec correction d'Aspin-Welsh, p=0.034). A noter que l'une des valeurs obtenues chez les chiennes malades a été jugée extrême (0.752) et a été ignorée lors du calcul de ces moyennes.

Ici aussi, l'implémentation d'une valeur seuil (égale à la valeur moyenne observée chez les témoins + 2 écarts-types) montre une probante distinction entre les deux groupes (test de Fisher, p=0.016).

2.3. Evaluation du niveau d'expression de la NGAL et de la MMP-9 par immunohistochimie

- Efficacité du marquage

43 sections de glande mammaire tumorale et 13 sections de glande mammaire ne présentant pas d'anomalie histologique ont été marquées. Dans la très grande majorité des sections, y compris celles de glandes saines, et aussi bien pour NGAL que pour MMP-9, il était possible d'observer au moins quelques cellules luminales qui présentaient un marquage positif, contrairement aux sections ayant servi de témoins négatifs (utilisation de sérum pré-immun en guise d'anticorps primaire) qui ne présentaient aucun signal positif.

Toutes les sections de glandes tumorales étaient positives, aussi bien à la NGAL qu'à la MMP-9. Les niveaux d'expression variaient le plus souvent non pas par la proportion de cellules positives (souvent entre 60 et 100%), mais par l'intensité du signal (allant de 1 à 3).

- Corrélation entre le niveau d'expression et le statut cancéreux

Les échantillons sains étaient dans la grande majorité négatifs à la MMP-9 (85%), sinon faiblement positifs (15%). Concernant la NGAL, 62% étaient négatifs et 38% faiblement positifs.

En comparaison, 100% des sections de glandes tumorales étaient faiblement ou fortement positives.

Pour la NGAL, le niveau d'expression est significativement corrélé au statut cancéreux (test de Fisher, p=0.001 si l'on compare les sains et les tumeurs bénignes, p=0.0001 si l'on compare les sains et les tumeurs malignes). Il en est de même pour la MMP-9 (test de Fisher, p=0.0001 si l'on compare les sains et les tumeurs bénignes ou les sains et les tumeurs malignes).

- Corrélation entre le niveau d'expression et le statut de malignité

Les sections de tumeurs malignes ont tendance à présenter un niveau d'expression de NGAL plus élevé que les témoins, mais la significativité n'a pu être démontrée (test de Fisher, p=0.13). La MMP-9 en revanche, est exprimée plus fortement en cas de tumeur maligne qu'en cas de tumeur bénigne (test de Fisher, p=0.04).

- Corrélation entre le niveau d'expression et d'autres facteurs pronostiques

Le niveau d'expression de la MMP-9 a tendance à augmenter avec le grade histologique des tumeurs malignes. En regroupant les grades II et III, l'expression au sein de ce groupe est significativement supérieure à celle observée dans les échantillons de grade I (test de Fisher, p=0.04). Ce n'est pas le cas pour la NGAL.

De plus, aucune corrélation n'a pu être démontrée comme significative entre le niveau d'expression des deux protéines et d'autres facteurs tels que :

-l'âge des patients
-le nombre de tumeurs
-le statut stérilisé ou non
-le temps de survie
-la présence de métastases dans les nœuds lymphatiques

- Corrélation entre les deux protéines

La NGAL étant capable de se lier de manière covalente à la MMP-9 et d'inhiber ainsi la dégradation de cette dernière, il semblait judicieux de voir si les niveaux d'expression de ces deux protéines étaient corrélés. Le test de corrélation de Spearman démontre que les deux protéines ont effectivement tendance à avoir des variations parallèles (p=0.0001) et ont un coefficient de corrélation r = 0.67.

A noter que pour 64% des échantillons analysés, les résultats (négatif, faiblement positif ou fortement positif) obtenus pour les deux protéines étaient identiques.

3. DISCUSSION

3.1. Optimisation du protocole de production des anticorps anti-MMP-9

- Protéine contaminante

Une étape d'expression préliminaire de l'antigène à petite échelle par les bactéries BL21 a été réalisée afin de déterminer les conditions optimales d'expression. Les conditions ainsi déterminées (37°C pendant 9 heures) présentaient cependant un inconvénient. En effet, une protéine légèrement plus grosse que la MMP-9-Dist était également produite de manière importante par les bactéries. Cette protéine était d'autant plus gênante qu'elle se liait elle aussi aux billes de sepharose lors de l'extraction et qu'il était donc impossible de s'en défaire.

Après plusieurs essais à grande échelle, il a été décidé d'opter pour de nouvelles conditions d'expression (25° pendant 24 heures), qui diminuaient de moitié la production de MMP-9-Dist mais n'entrainaient qu'une expression marginale de la protéine contaminante.

- Efficacité de l'étape d'extraction

La principale difficulté rencontrée lors de la production de l'antigène concernait l'étape d'extraction. En effet, la protéine se liait très peu aux billes de sépharose et rendait son extraction très peu efficace lors des premiers essais. Après plusieurs tentatives, il a été décidé de modifier la composition de la solution tampon utilisée en augmentant de manière importante les conditions dénaturantes (les concentrations en urée et en NaCl ont été augmentées respectivement de 6M à 8M et de 0.5M à 1M) et en réalisant l'étape de fixation à 4°C au lieu de 37°C.

- Impact sur l'immunisation

Les deux difficultés suscitées ont eu pour conséquence l'utilisation d'une solution antigénique peu concentrée (150 µg/ml) et présentant un taux de pureté moyen (70%) lors de la primo-injection et du premier rappel. Les nouvelles conditions d'expression et d'extraction ont cependant permis d'obtenir des solutions très concentrées (400-1000 µg/ml) et très pures (99%) pour les rappels suivants.

3.2. Expression de la NGAL dans l'urine et le sérum

- Représentativité de l'échantillon

Si le nombre d'échantillons sanguins était suffisamment conséquent (n=28), celui d'échantillons d'urine était par contre relativement faible (n=8) et ne permet qu'une étude préliminaire méthodologique de l'évaluation de l'expression de la NGAL. Les résultats obtenus étant encourageants, il serait intéressant de répéter cette étude sur un grand nombre d'échantillons.

- Reproductivité des essais

Le nombre d'échantillons sanguins (n=42 en comptant les témoins sains) ne permettaient pas de les tester tous sur une seule plaque ELISA. Cependant, les essais ont été répétés et le biais inter-essais a été jugé négligeable.

- Mise au point d'un test sandwich ELISA pour la MMP-9

Le test ELISA permettant d'évaluer l'expression de la MMP-9 n'a pas pu être mis au point, principalement en raison des difficultés rencontrées lors de la production de l'antigène. Celui-ci n'était pas extrait en quantité suffisante pour immuniser des lapins en plus des souris. Après modification des conditions d'expression et d'extraction il a été possible d'immuniser deux lapins qui permettront à une étude future de mettre au point un tel test.

- Expression urinaire et sérique de la NGAL et tumeur mammaire

Le niveau moyen d'expression dans le sang des sujets atteints n'était pas significativement supérieur à celui des témoins. Cependant, il est intéressant de noter qu'en médecine humaine, cette différence était très faible (Provatopoulou et al, 2009), et qu'un nombre d'échantillons suffisamment important serait peut-être capable de rendre cette différence significative. L'application d'une valeur seuil montre qu'il semble tout de même y avoir un lien entre le statut cancéreux et le niveau d'expression de la protéine.

Concernant le niveau d'expression urinaire, il a été montré que la différence était significative et que l'expression de la NGAL pouvait donc être corrélée au statut cancéreux. Ces résultats sont en correspondance avec ceux obtenus en médecine humaine. Cependant, comme il a été mentionné précédemment, le faible nombre d'échantillons affaiblit clairement la puissance statistique du test et il serait intéressant, à présent que la méthode est au point, de réitérer cette étude avec un nombre plus important d'échantillons.

3.3. Expression de la NGAL et de la MMP-9 dans le tissu mammaire

- Optimisation du protocole

Les principaux paramètres à avoir été optimisés étaient :

-la puissance du four à micro-ondes lors de la révélation des antigènes : testée de 600W à 1000W, la puissance de 800W a semblé offrir le meilleur compromis entre efficacité de révélation et faible dégradation du tissu.

-réactifs de blocage : le sérum de chèvre et l'H_2O_2 ont été préférés au réactif commercial fourni dans le kit *Dako*™ qui offrait un moins bon contraste.

-dilution et temps de contact du premier anticorps : lors de l'optimisation du protocole, la dilution a été doublée à plusieurs reprises jusqu'à ce que l'augmentation affecte la qualité du marquage. Nous avons pu en déduire que les dilutions de 1:400 pour la NGAL et de 1:100 pour la MMP-9 permettaient d'économiser au maximum les anticorps sans affecter la qualité du marquage. De même, divers temps de contact ont été testés (1h, 2h, 4h, une nuit). Nous avons opté pour 2h mais ce paramètre n'impactait que très peu le résultat.

-dilution du DAB et temps de contact : de façon similaire, les temps de contact et la concentration du DAB ont été augmentés jusqu'à ce que cela affecte la qualité du marquage ou jusqu'à ce que cela n'augmente plus le contraste.

- Limites et reproductibilité

Représentativité des échantillons : il est intéressant de noter que la répartition des types de tumeurs n'est pas représentative de l'épidémiologie de cette affection. Les tumeurs bénignes ne représentaient en effet que 26% de nos échantillons. De plus, le faible nombre de tumeurs bénignes (n=11) impacte aussi l'étude statistique. Il serait donc intéressant de réitérer l'expérience avec un plus grands nombre de tumeurs bénignes. Enfin, le faible nombre d'échantillons présents dans chaque sous catégorie de différents facteurs pronostiques ne permet pas de conclure quant à une éventuelle corrélation entre les niveaux d'expression et ces marqueurs.

Représentativité des témoins : contrairement aux études déjà réalisées sur la MMP-9 canine et son homologue humaine, nous n'avons pas utilisé de glandes saines de patients sains, mais des glandes apparemment saines de patients atteints de tumeur mammaire sur une autre mamelle. Connaissant le réseau artériel, veineux et lymphatique qui peut relier les mamelles entre elles, il n'est pas exclu que la présence d'une tumeur puisse avoir une répercussion sur l'expression des protéines dans les mamelles saines. Ceci pourrait notamment expliquer que des résultats faiblement positifs ait été obtenu sur des tissus sains, contrairement aux études antérieures.

Enfin, un autre paramètre pouvant influencer l'exactitude ou la reproductibilité des résultats obtenus est la fiabilité du système de score. En plus de l'aspect subjectif de ce système, le

score a été déterminé par deux observateurs qui ne sont pas des spécialistes en anatomie pathologique (deux vétérinaires dont un actuellement en résidanat d'anatomie pathologique).

- Relation entre l'expression tissulaire de la MMP-9, le statut tumoral et certains facteurs pronostiques

En accord avec ce qui a pu être mis en évidence chez la femme dans les études réalisées par Hirayama et al. en 2002 et chez la chienne dans les études de Santos et al. en 2012, nos résultats montrent que le niveau d'expression de la MMP-9 dans le tissu mammaire de chiennes atteintes de tumeur peut être un bon marqueur de cette pathologie. Son expression est aussi impactée par le statut de malignité de la tumeur, ainsi que par son grade histologique si cette dernière est maligne. Cependant la différence entre grade I et grade II n'a pu être démontrée comme étant significative, probablement en raison du faible nombre d'échantillons présents dans ces deux catégories.

Contrairement aux découvertes récentes de Santos et al., l'expression de la protéine n'a pas pu être reliée à d'autres facteurs pronostiques. Le faible nombre de patients ayant présenté des métastases (n=5) d'une part et le caractère récent des exérèses (réalisées en 2011 et 2012) d'autre part, ont probablement été un obstacle dans l'étude de la corrélation éventuelle existant entre la MMP-9 et la présence de métastases ou la baisse du temps de survie.

A noter qu'il s'agit de la première étude visant à évaluer le niveau d'expression de la MMP-9 par immunohistochimie avec des anticorps anti MMP-9 canine. En effet, les études réalisées précédemment, notamment par Santos et al en 2012, ont été menées avec des anticorps anti MMP-9 humaine.

- Expression de la NGAL dans le tissu mammaire de chiennes atteintes de tumeur

Cette étude est la première à s'être intéressée à l'expression tissulaire de la NGAL dans le cas de tumeur mammaire. Nos résultats montrent, en accord avec les découvertes réalisées en médecine humaine par Bauer et al en 2008, que le niveau d'expression de la NGAL est significativement augmenté en cas de tumeur. Cependant, ces résultats ne permettent pas de mettre en évidence un effet du statut malin ou bénin sur cette expression. Aucun autre facteur pronostique ne semble impacter le niveau d'expression mais une telle étude nécessiterait un nombre beaucoup plus conséquent d'échantillons afin d'être concluante.

- Perspectives

Dans un futur proche, le Graduate Institute of Microbiology and Public Health de la Chung Hsing University de Taichung, Taiwan, se penchera sur la mise en place, à partir de l'antigène produit lors de cette étude, d'un test sandwich ELISA apte à évaluer le niveau d'expression de la MMP-9 canine dans l'urine et le sérum de chiennes atteintes de tumeur mammaire.

Un test ELISA permettant la mise en évidence du complexe MMP-9/NGAL sera aussi réalisé. En effet, des études en médecine humaine (Provatopoulou et al., 2009) ont montré que ce complexe pouvait être un biomarqueur parfois plus efficace que les protéines étudiées individuellement.

L'augmentation significative du niveau d'expression de la NGAL dans l'urine de chiennes atteintes suggère que si une étude réalisée à plus grande échelle tendait à confirmer nos résultats, la NGAL pourrait être un biomarqueur intéressant dans le diagnostic non invasif de tumeur mammaire. Cependant, au vu de nos résultats préliminaires, un tel test serait plutôt un test d'exclusion car sa spécificité semble bien meilleure que sa sensibilité. Un plus grand nombre d'échantillons rendrait de plus possible l'étude de la corrélation entre le niveau d'expression et les facteurs pronostiques les plus couramment suivis.

Les résultats obtenus en immunohistochimie sont très encourageants. Ils démontrent que la NGAL et la MMP-9 sont d'intéressants marqueurs tissulaires de la pathologie. De plus, en s'appuyant sur les résultats obtenus en médecine humaine par Fernandez et al. en 2005, il est possible que ces deux protéines soient impliquées dans la progression de ces tumeurs, en particulier la MMP-9 pour qui le niveau d'expression a pu être corrélé avec le statut de malignité et le type histologique.

Si d'autres études venaient à confirmer les résultats obtenus lors de nos essais, une des problématiques suggérées serait de savoir si l'inhibition de ces protéines pourrait ralentir la progression et la dissémination de la tumeur et dans l'affirmative, comment pourrait-elle être ciblée de manière thérapeutique. Il n'existe encore aucun inhibiteur thérapeutique de la NGAL mais des inhibiteurs de la MMP-9 ont déjà été testés chez la femme. Si les premiers tests réalisés sur des modèles murins ont donné des résultats très prometteurs (Wojtowicz-Praga et al., 1997), les premiers essais cliniques ont en revanche donné des résultats moins encourageants (Pavlaki et al., 2003).

Enfin, le cancer du sein de la femme et les tumeurs mammaires chez la chienne partagent de nombreuses similitudes : une origine histologique similaire, un même type d'extensions métastatiques régionales et générales, un âge d'apparition comparable (proportionnellement à l'espérance de vie) et de nombreux autres points communs. Il s'agit aussi du type de tumeur le plus prévalent chez les deux espèces (Hill et al, 2005). Le fait que les résultats obtenus lors de l'évaluation du niveau d'expression de la NGAL et de la MMP-9 soient en accord avec ceux obtenus en médecine humaine renforce cette similitude et suggère que les tumeurs mammaires de la chienne pourraient éventuellement servir de modèle spontané dans la recherche de moyens thérapeutiques agissant via la modulation de l'expression de ces protéines.

CONCLUSION

Les tumeurs de la glande mammaire représentent l'un des groupes de tumeur le plus étudié en recherche vétérinaire canine, principalement du fait de son haut niveau de prévalence. Les études enquêtant sur les protéines surexprimées en présence de phénomènes cancéreux sont essentielles. En effet, non seulement ces dernières peuvent servir de bio-marqueurs dans le diagnostic, l'évaluation ou le suivi de ces pathologies, mais elles peuvent aussi parfois offrir de nouvelles solutions thérapeutiques s'il est démontré qu'elles présentent des effets favorisant la croissance, l'expansion ou la dissémination des tumeurs.

En s'inspirant des études réalisées en médecine humaine sur la MMP-9 et la NGAL, qui ont à plusieurs reprises prouvé une corrélation indéniable entre les niveaux d'expression de ces deux protéines et le statut cancéreux, nous avons cherché à évaluer leur niveau d'expression dans les mêmes types d'échantillons que ceux étudiées en médecine humaine (sérum, urine et tissu mammaire).

Les niveaux d'expression sérique et urinaire de la NGAL ont été évalués pour la première fois à l'aide d'un test sandwich ELISA fait maison tandis que son expression tissulaire a été étudiée par immunohistochimie en utilisant des anticorps spécifiques de l'espèce canine. Il s'agit également de la première étude décrivant la production et l'utilisation d'anticorps anti MMP-9 canine dans des tests immunohistochimiques.

Nos résultats démontrent une corrélation significative entre l'expression de la NGAL et celle de la MMP-9 dans le tissu mammaire et leur niveau d'expression est un marqueur efficace du statut tumoral. De plus, l'expression de la MMP-9 a pu être reliée à certains facteurs pronostiques tels que la malignité ou le grade histologique. Enfin, il a été montré que l'expression de la NGAL dans le sérum et l'urine était plus élevée chez les chiennes atteintes de tumeur mammaire.

Ces résultats sont cohérents avec les découvertes réalisées en médecine humaine, et confirment la similarité entre les tumeurs de la glande mammaire de ces deux espèces. Connaissant les propriétés de ces protéines (notamment la capacité de la MMP-9 à dégrader la matrice extracellulaire, favorisant ainsi l'expansion et la dissémination de la tumeur), ces résultats suggèrent par ailleurs que l'utilisation d'inhibiteurs de la MMP-9 (ou éventuellement de la NGAL) pourrait être envisagée en tant que possible option thérapeutique contre la progression, l'infiltration et la dissémination des tumeurs mammaires.

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ECOLE NATIONALE VETERINAIRE

Université Paul Sabatier
TOULOUSE III

AGREMENT SCIENTIFIQUE

En vue de l'obtention du permis d'imprimer de la thèse de doctorat vétérinaire

Je soussigné, ***DELVERDIER Maxence***, Enseignant-chercheur, de l'Ecole Nationale Vétérinaire de Toulouse, directeur de thèse, certifie avoir examiné la thèse de ***BELOT Guillaume*** intitulée « ***Evaluation of the expression of matrix metalloproteinase 9 and neutrophil gelatinase associated lipocalin in serum, urine and tumoral tissues of female dogs suffering from mammary gland tumors*** » et que cette dernière peut être imprimée en vue de sa soutenance.

Fait à Toulouse, le 20 Décembre 2012
Professeur DELVERDIER Maxence
Enseignant chercheur
de l'Ecole Nationale Vétérinaire de Toulouse

Vu :
Le Directeur de l'Ecole Nationale Vétérinaire de Toulouse
Professeur Alain MILON

ECOLE NATIONALE VETERINAIRE DE TOULOUSE

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a validé son année d'approfondissement le : 18/10/2012
n'a plus aucun stage, ni enseignement optionnel à valider.

Université de Toulouse

TITLE: EVALUATION OF THE EXPRESSION OF MATRIX METALLOPROTEINASE 9 AND NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN IN SERUM, URINE AND TUMORAL TISSUES OF FEMALE DOGS SUFFERING FROM MAMMARY GLAND TUMORS

ABSTRACT

In human medicine, it has been shown recently that the level of expression of matrix metalloproteinase-9 (MMP-9) and neutrophil gelatinase-associated lipocalin (NGAL) in serum, urine, and breast tissue were significantly increased in patients with breast cancer and correlated to several prognostic factors.

In the first part, the author presents the MMP-9 and NGAL as a synthesis of current knowledge on their ability to serve as biomarkers of breast cancer in women.

In the second, the author presents the production of canine MMP9 and NGAL recombinant proteins, the production of polyclonal antibodies, and their use in various techniques (ELISA, western blot, immunohistochemistry) to assess the level of expression of these proteins in the blood, urine and breast tissue of dogs suffering from mammary gland tumors and demonstrate a positive correlation between these proteins and the presence of a tumor disease of the mammary gland.

KEY WORDS

MAMMARY GLAND TUMOR - CANINE - MMP9 - NGAL - BIOMARKER - IMMUNOHISTOCHEMISTRY - ELISA

AUTHOR

BELOT GUILLAUME

TITRE : EVALUATION DE L'EXPRESSION DE LA MATRIX METALLOPROTEINASE 9 ET DE LA NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN DANS LE SERUM, L'URINE ET LE TISSU TUMORAL DE CHIENNES ATTEINTES DE TUMEURS MAMMAIRES

RESUME

En médecine humaine, il a été montré récemment que le niveau d'expression de la matrix métalloprotéinase-9 (MMP-9) et de la neutrophil gelatinase-associated lipocalin (NGAL) dans le sérum, l'urine, et le tissu mammaire étaient significativement augmentés chez les patientes atteintes de cancer du sein et étaient corrélés à de nombreux facteurs pronostiques.

Dans une première partie, l'auteur présente la MMP-9 et la NGAL ainsi qu'une synthèse des connaissances actuelles portant sur leurs aptitudes à servir de biomarqueurs du cancer du sein chez la femme.

Dans la seconde, l'auteur présente la production de MMP9 et de NGAL canines recombinantes, la production d'anticorps polyclonaux associés, et leur utilisation dans diverses techniques (ELISA, western blot, immunohistochimie) permettant d'évaluer le niveau d'expression de ces protéines dans le sang, l'urine et le tissu mammaire de chiennes malades et de démontrer ainsi une corrélation positive entre ces protéines et la présence d'une affection tumorale de la glande mammaire.

MOTS CLES

TUMEURS MAMMAIRES - CHIENNE - MMP9 - NGAL - BIOMARQUEUR - IMMUNOHISTOCHIMIE - ELISA

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