Studies on the Pathogenesis and Management of Prostate Carcinoma in Dogs

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Studies naar de pathogenese an behandeling van prostaat carcinoom bij de hond

(met een samenvatting in het Nederlands)

Proefschrift

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Door

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CHAPTER 1

GENERAL INTRODUCTION

MORPHOLOGY AND PHYSIOLOGY OF THE CANINE PROSTATE

The prostate is the only major accessory sex gland in the dog. It is a bilobed structure that completely encircles the proximal portion of the urethra. In young animals, the prostate lies entirely within the pelvic cavity but with sexual maturity it increases in size and assumes a more abdominal position. Histologically, the prostate has an epithelial and a stromal component (**Figure 1**). The epithelial cells of the secretory region form tubuloaveoli that drain through ducts into the urethra. The prostate is surrounded by a capsule containing smooth muscle fibers that extend into the organ, dividing the alveolar tissue into distinct lobules. Embryologically the prostate develops from several groups of outgrowths of the urethral epithelium on either side of the entrance of the vas deferens.

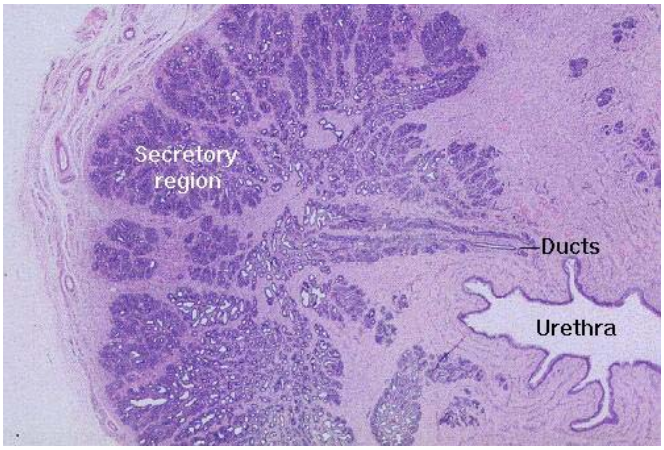

Figure 1. Histological structure of the prostate

Blood supply to the prostate is by the prostatic artery, which arises from the pudendal or umbilical artery. Anastomoses can be found between the prostatic arteries and the urethral artery, and the cranial and caudal rectal artery. Venous blood returns via the prostatic vein to the internal iliac vein. Lymph drains into the iliac lymph nodes. Sympathetic innervation is provided by the hypogastric nerve, which stimulates the active secretory process and expulsion of secretions by smooth muscle contractions. Parasympathetic innervation with fibers from the pelvic nerve also contributes to smooth muscle contraction (Basinger, 2003). Fluid secreted by the prostate forms more than 90% of the total volume of the ejaculate (Johnston et al., 2000) and is expulsed in the first and third fractions of the ejaculate. The main functions of prostatic fluid are to reduce the viscosity of the ejaculate and to facilitate sperm transport. Prostatic secretions are acidic and have a bactericidal effect, which may help to prevent ascending urinary tract infections.

DISEASES OF THE CANINE PROSTATE

With increasing age and under the influence of androgens the prostate undergoes spontaneous enlargement referred to as benign prostatic hyperplasia.
Several different diseases of the prostate occur in middle-aged and older dogs and include benign hyperplasia, prostatitis, prostatic abscess, prostatic and para-prostatic cysts, prostatic

metaplasia and prostatic neoplasia. With the exception of prostatic neoplasia, prostatic disease generally affects intact male dogs (Basinger & Rawlings, 1987; Basinger, 2003).
This present thesis concentrates only on prostate carcinoma (PCA).

PROSTATE CARCINOMA (PCA)

The dog is the only species other than man that spontaneously develops prostate cancer regularly. It has therefore been considered as a model for studying pathogenesis (Winkler et al., 2005; Winkler et al., 2006), treatment (Huang et al., 2005; Janzen et al., 2005; Levy et al., 1999; Liu et al., 2006; Nau et al., 2005) and prevention (Lamb & Zhang, 2005; Waters et al., 2003) of prostate cancer in people. For this reason, the following overview will focus in each subsection on similarities and differences between human and canine PCA.

Epidemiology

Incidence and age

Tumours of the prostate are not commonly seen in dogs. Prevalence reported from necropsy studies is thought to be between 0.2% and 0.6% (Bell et al., 1991). Usually old dogs are affected with an average age of occurrence of 10 years (Cooley & Waters, 2001; Dorfmann & Barsanti, 1995) and it has been shown that castrated males have a greater risk of developing prostatic carcinoma than intact male dogs (Teske et al., 2002).
In people, prostate cancer also occurs in older individuals. Prostate cancer rarely occurs before the age of 50, but passed that age, the incidence increases rapidly. It is thought that more than 60% of men over 80 have prostate cancer even though the disease is usually not clinically apparent (Bostwick et al., 2004). However, unlike in dogs, prostate cancer occurs commonly in men. In fact, it is the most common cancer in men (Edwards et al., 2005) and a third of all new cancer cases each year are cancers of the prostate. Although it is not the most deadly type of cancer in people, it nevertheless accounts for more than 30’000 deaths in the United States of America each year (Jemal et al., 2005) and represents therefore a major public health issue.

Breed

In dogs, a retrospective study at the University of Utrecht revealed that the Bouvier des Flandres had an almost 8.5 times higher risk of developing the disease than other breeds (Teske et al., 2002). This is the only mention of a difference of incidence between breeds in dogs. Otherwise middle to large breed dogs seem to be overrepresented (Cooley & Waters, 2001).
In humans there is a well documented difference in incidence of PCA between different ethnic groups (Bostwick et al., 2004; Winters et al., 2001). African-American men have the highest incidence of prostate cancer in the world (Bostwick et al., 2004; Jemal et al., 2005). However, it is uncertain whether this difference is truly due to ethnicity as a number of other factors may also explain the predisposition of a certain group to PCA. These include differences in exposure (particularly dietary differences), differences in detection and genetic differences (Bostwick et al., 2004). Only the latter should be of importance in dogs, since

there are no breed-related differences in nutrition and veterinary treatment in the dog population of the Netherlands. In humans, differences in serum concentrations of androgens may explain the racial differences in prostate cancer risk. In African-American men higher concentrations of testosterone and sex hormone-binding globulin (SHBG) have been found, compared to the Caucasian population (Abdelrahaman et al., 2005; Winters et al., 2001). It has been hypothesized that SHBG could activate androgen responsive genes (Winters et al., 2001). Other race-related differences reported include the activity of the enzyme 5α-reductase (Ross et al., 1992; Wu et al., 2001), concentrations of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (Platz et al., 1999; Winter et al., 2001) as well as differences in the microsatellite alleles at the androgen receptor (AR) locus. In particular the length of CAG repeats in these microstallite alleles may be a risk factor for the development of PCA (Coetzee & Ross, 1994; Giovannucci et al., 1997). This is supported by the fact that African-American men were found to have shorter CAG repeats (Platz et al., 2000) and Japanese men longer CAG repeats (Ekman et al., 1999) compared to white men.

Pathology and natural behaviour

In the dog all prostatic tumours reported are malignant and the majority have been reported to be adenocarcinomas (Cooley & Waters, 2001; Theilen & Madewell, 1979). In some cases transitional cell carcinomas are seen originating from the collecting ducts of the prostate. Canine prostate carcinomas are very malignant tumours and metastases are commonly present at the time of diagnosis. The main sites of metatstatic spread are the sublumbar lymph nodes, lungs and skeletal system, particularly the lumbar vertebrae (**Figure 2**), pelvis and femur (Bell et al., 1991; Cornell et al., 2000).

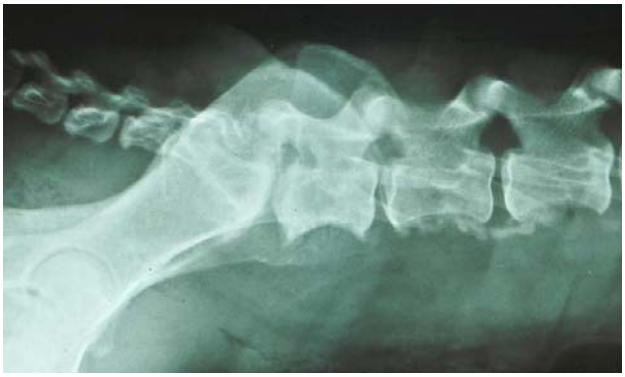

Figure 2. Metastases of PCA to the skeleton. Note the bone proliferation on the ventral aspect of the lumbar vertebrae

Canine PCA originates from urothelial/ductal cells rather than from acinar cells as is the case in humans (Leav et al., 2001). Since the growth of ductal cells in not dependent on androgens, this may explain the fact that canine PCA may develop in the absence of androgens (Sorenmo et al., 2003). Patterns of histological differentiation vary according to authors (**Table 2**).

Table 2. Histological classification of canine PCA

(Leav & Ling, 1968)	(Young et al., 2000)	
Type I Intra-alveolar proliferation	Micropapillary	Multiple foci of irregularly shaped alveoli with fronds of neoplastic cells radiating from a dense basal layer toward a central lumen
Type II Small acinar	Small acinar	Tumor cells form small acini imbedded in proliferating stromal tissue. Desmoplastic response may be prominent. Resembles acinar atrophy.
Type III Syncytial	Sarcomatoid	Tumors composed of pleomorphic, often fusiform cells, frequently arranged in sheets, and resembling a sarcoma. These cells are intermingled with neoplastic acini.
Type IV Discrete epithelial		Singular, discrete, round to polygonal (often "signet ring") epithelial cells embedded in stroma
Type V Poorly differentiated	Ductal Solid	Sheets, nests, and cords of neoplastic cells with little or no tendency to form glandular structures.

(Cornell et al., 2000)
Adenocarcinoma
Urothelial carcinoma
Squamous cell carcinoma
Mixed morphology: 2 or more of the following types: - glandular - urothelial - squamoid - sarcomatoid

Canine PCA is a histologically heterogenous tumour often featuring more than one histological type (Cornell et al., 2000; Waters et al., 1998). The intra-alveolar pattern appears to be the most common (Bell et al., 1991; Leav et al., 2001), however there is a tendency for less differentiated tumour to be more frequent in castrated dogs, compared to intact males (Cornell et al., 2000). This correlates with the situation in humans, where androgen ablation often leads to the development of less well differentiated PCA (higher Gleason grades) (Algarte-Genin et al., 2004).

As in humans, high-grade prostate intraepithelial neoplasia (HG-PIN) has been found in the prostate of dogs with and without prostate carcinoma. In humans it is thought that HG-PIN is

a precursor of prostate cancer. Histological characteristics of HG-PIN are disruption if the basal membrane, increased proliferative capacity and microvessel density (Waters & Bostwick, 1997). The presence of HG-PIN on prostatic biopsy in humans is the most significant risk factor for subsequent prostate carcinoma (Waters & Bostwick, 1997). HG-PIN was present in 66% to 72% of dogs with spontaneous prostate carcinoma (Aquilina et al., 1998; Waters & Bostwick, 1997), however in a more recent study HG-PIN was only found in 7% of prostate carcinoma (Cornell et al., 2000). In dogs without prostate carcinoma HG-PIN was identified in 3% of the cases in one study (Aquilina et al., 1998) and was not found at all in another (Madewell et al., 2004). The role of HG-PIN as a precursor of PCA in dogs is therefore unclear and it seems unlikely that its detection in dogs without PCA can be used as a screening for the early stages of PCA in dogs, like it is in people.

Aetiology and pathogenesis

Androgens

Androgens are known to stimulate growth of prostate cells in both humans and dogs (Niu et al., 2001; Niu et al., 2003). In humans, not only normal prostate cells but also PCA cells are highly dependent on androgen for growth and Huggins and Hodges recognised already in the early 1940s that castration was an effective treatment for PCA (Huggins & Hodges, 2002). Today, hormone ablation therapy is still the mainstay of systemic treatment for PCA in humans. In most cases however, androgen ablation fails after a period of time and PCA progresses to a hormone-independent state (Denmeade and Isaacs, 2002; Taplin and Balk, 2004; Linja and Viskorpi, 2004).

In dogs, the aetiologic role of androgens is less clear since PCA has a higher prevalence in castrated animals than in intact males (Bell et al., 1991; Obradovich et al., 1987; Teske et al., 2002). There appears to be no relationship between the age at castration and the age at the development of PCA (Obradovich et al., 1987; Teske et al., 2002), suggesting that castration does not initiate the development of PCA in dogs but might be a promoting factor. In addition, in intact male dogs with PCA androgen ablation does not have any anti-tumour effect. As the histomorphology of the canine PCA is often less differentiated, canine PCA thus resembles the hormone-independent state of human PCA. One of the possible explanations for the development of PCA in the absence of androgens is the fact that canine PCA appears to originate from basal cells in the duct areas rather than from acinar cells (Leav et al., 2001). Castration causes a massive decrease in the in the number of epithelial cells but an increase in the population of basal or stem cells that are not dependent on androgens for proliferation (Mahapokai et al., 2000).

Androgen receptor

The normal development of the prostate is dependent on androgens binding to and acting through the androgen receptor (AR) (Roy et al., 1999). The AR is also expressed in PCA cells, both in hormone-sensitive and hormone-resistent PCA. However AR expression is heterogeneous, meaning that some PCA cells lose AR expression. The reasons for this heterogeneity are unclear (Heinlein and Chang, 2004). The progression of cancer in the

absence of androgens does not rule out that the AR may play a role in the pathogenesis of PCA. In the absence of androgens the AR signalling may still be active but altered (Scher and Sawyers, 2005).
The AR may still be directly activated by ligands that are not influenced by hormonal therapy. For instance detectable levels of androgen remain in PCA tissue even after castration (Geller, 1991; Labrie et al., 1983; Mizokami et al., 2004). In addition, production of androgens by the adrenal gland is not stopped by traditional androgen ablation treatments such as surgical castration. Specific blockade of adrenal androgens in humans has been shown to have an effect against PCA and suggests that AR activation by adrenal androgens may be of importance in PCA progression despite androgen ablation (Trachtenberg and Pont, 1984; Harnett et al., 1987). It is not known whether intratumoural androgens come from an adrenal source or whether they are synthesised directly within the tumour by an intracrine mechanism (Scher and Sawyers, 2005; Labrie et al., 1993).
The AR may also be amplified, thereby increasing the number of receptors (Scher and Sawyers, 2005). In this case the tumour is sensitised and reacts to lower levels of circulating androgens. Or the AR may even mutate and become sensitive to other ligands such as other steroid hormones (Scher and Sawyers, 2005; Chen et al., 2005).
Alternatively, the AR may be activated indirectly by a change in the expression of coactivators or corepressors mediating AR activity (Scher and Sawyers, 2005). These may sensitise the receptor to lower concentrations of ligand, sensitise it to other ligands or cause an activation in the absence of ligands (Scher and Sawyers, 2005).

Androgen receptor gene
Variations in the sequence of the AR gene have been shown to influence the activity of the receptor as well as the risk to develop PCA (Heinlein and Chang, 2004). The human AR gene is located on the X chromosome and spans approximately 90 Kb of genomic DNA. It is composed of eight exons that encode three major domains: an N-terminal transcriptional activation domain encoded by exon one, a central DNA-binding domain encoded by exons two and three and a C-terminal steroid binding domain encoded by exons four to eight (Culig et al., 2002). The N-terminal transactivation domain of the AR gene in humans contains 3 polyglutamine repeats and 1 polyglycine repeat. These polyglutamine and polyglycine repeats are encoded by a CAG repeat and a GGN repeat respectively (Choong et al., 1998, Chen et al., 2002). The first CAG repeat length (commonly referred to as CAG-I) as well as the GGN repeat length are polymorphic in the human population. The second CAG repeat (CAG-II) and third CAG repeat (CAG-III) are not polymorphic and have a length of 6 and 5 contiguous repeats, respectively (Lubahn et al., 1988b, Choong et al., 1998).
The polymorphic CAG-I repeat is uninterrupted, varies between eleven and thirty-one contiguous repeats in length and ends with CAA (Edwards et al., 1992). The length of this polymorphic polyglutamine tract in men has been linked to various diseases, including two that affect the prostate gland (Nelson et al., 2002). First, Giovannucci et al. (1999) found that men with CAG repeats less than 19 long are at a significantly greater risk for developing benign prostatic hyperplasia than those with CAG repeats more than 25 in length. Second, Coetzee and Ross (1994) were the first to suggest an inverse correlation between the

occurrence of prostate cancer in men and the number of CAG repeats in the androgen receptor gene. The findings of Irvine et al. (1995) and Heinlein et al. (2004) underline this suggestion: these investigators found that ethnic differences in prostate cancer susceptibility are inversely correlated with the predominant CAG repeat length in each group. On average, Asians have the lowest incidence of prostate cancer and the longest CAG repeat lengths, whereas African-Americans have the highest incidence of prostate cancer and the shortest CAG repeat length. Further indications for a relationship between CAG repeat length in the AR and prostate cancer risk in men were found in patient-control studies. Giovannucci et al. (1997), Hakimi et al. (1997) and Ingles et al. (1997) reported that short CAG repeats were more common among men with prostate cancer than among controls. Several in vitro studies that have shown that CAG repeat length and transactivation of the AR are inversely correlated (Feldman, 1997, Coetzee and Ross, 1994): i.e. a shorter CAG repeat length increases AR transcriptional activity (Chamberlain et al., 1994). This may be a functional link between the CAG repeat length and the incidence of prostate cancer and may imply that increased AR transcriptional activity increases the risk of prostate cancer development.

The second polymorphic AR trinucleotide repeat, the GGN repeat, varies between ten and thirty contiguous repeats in length and is composed of a consensus sequence that resembles $GGT_{(3)}$-GGG-$GGT_{(2)}$-$GGC_{(n)}$ (Platz et al., 1998). This GGN repeat is not as well studied as the CAG repeat (Heinlein et al., 2004). Moreover, while some of these studies found a positive correlation between short GGN repeat lengths and prostate cancer risk (Stanford et al., 1997, Hakimi et al., 1997), others failed to identify this link (Edwards et al., 1999, Correa-Cerro et al., 1999). Functional studies also show ambiguous results: deletion of the GGN tract results in either an increased or a decreased AR activity or no alteration at all (Jenster et al., 1994, Gao et al., 1996). Extension of the repeat length to 48 GGN repeats has resulted in inhibition of AR activity (Chen et al., 2002). So far, no comparisons of varying GGN repeat length and AR transcriptional activity have been made (Heinlein et al., 2004).

Similar to the human AR and those reported for other species (e.g. rat and mouse), the canine AR sequence contains 3 polyglutamine repeats and 1 polyglycine repeat (Lu et al., 2001). In 1993, Shibuya et al. reported two of these $(CAG)_n$ microsatellites in the canine AR to be polymorphic. The first polymorphic CAG repeat (CAG-I) was found in a position similar to the human polymorphic CAG-I repeat and consisted of 10-12 consecutive CAGs, whereas the second polymorphic CAG repeat (CAG-III) was located at a position corresponding to that of the CAG-III repeat in the rat and resembled $CAG_{(10-13)}$-CAA-CAG-CAA-$CAG_{(6)}$-CAA-$CAG_{(2)}$. The canine CAG-II repeat has not been found to be polymorphic and has a length of 7 contiguous repeats (Lu et al., 2001). The occurrence of the CAG repeat polymorphisms in the canine AR gene has not been confirmed yet, nor have the lengths of these polymorphic CAG repeats ever been related to canine prostate cancer susceptibility.

The polyglycine repeat in the dog is characterized by eight glycine residues interspersed by 2 serines, an alanine and an asparagine in this region. In this respect, it differs from other species' polyglycine repeats as all other reported mammalian polyglycine repeats consist exclusively of glycine residues. The length of this repeat varies between species: 5 in the rat and mouse and 10-30 in humans. There have been no reports about variations in the length of the GGN repeat in the canine AR sequence (Lu et al, 2001).

Chromosome polysomy

Chromosome polysomy such as trisomy is the consequence of failed chromosomal disjunction and is the manifestation of genetic instability that has been implicated in the pathogenesis of many types of cancer including leukaemia, breast cancer and prostate cancer (Mark et al., 1999). In humans, trisomy of chromosome 7 appears to occur frequently in patients with PCA (Mark et al., 1999) and gain in chromosome 8 seems to correlate with the aggressiveness of PCA (Steiner et al., 2002). Similar karyotype changes have been described in a canine cell line derived from PCA (Winkler et al., 2005) as well as polysomy of chromosome 13 in a dog with PCA (Winkler et al., 2006). It is thought that these chromosomal aberrations may be one of the first steps in the carcinogenesis of PCA as they may result in an extra dose of oncogenes, a genetic imbalance that may lead to cancer (Mark et al. 1999).

Apoptosis

Apoptosis is an effective intrinsic anti-cancer mechanism with which a tissue disposes of transformed cells. Resistance to signals inducing apoptosis may favour accumulation of cells and lead to the establishment of early neoplastic lesions (Bostwick et al., 2004). Derangement of apoptosis seems to play a key role in the pathogenesis of prostate cancer (Tang et al., 1997; Colombel et al., 1996). The gene *bcl-2* is considered to be an apoptosis suppressor gene. Over-expression of the protein linked to this gene may block or delay the onset of apoptosis. Although the expression of *bcl-2* varies in human PCA, it is expressed consistently in PCA cells after androgen ablation therapy and in hormone-independent tumours (Bostwick et al., 2004). This supports the theory that *bcl-2* is causally linked to apoptotic resistance in PCA cells.

Cyclooxygenase-2

Much attention has been directed recently towards the role of cyclooxygenase-2 (COX-2) in the pathogenesis of various types of cancer, including prostate carcinoma. Cyclooxygenase is the enzyme responsible for the synthesis of prostaglandins. Several isoforms of this enzyme exist and COX-2 is the inducible form. While COX-1 is constitutively expressed in many tissues, COX-2 expression is normally low or absent in most normal tissues. However COX-2 has been found to be expressed in many types of inflammatory and neoplastic diseases (Zha et al., 2004). The COX-2 enzyme itself and its RNA expression have been detected in several forms of human and canine cancer (Dubois et al., 1998). In the dog, these include oral squamous cell carcinoma, mammary tumors, transitional cell carcinoma of the urinary bladder and prostate carcinoma (Sorenmo et al., 2004; Gupta et al., 2000; Hussain et al., 2003; Mohammed et al., 2004; Khan et al., 2000; Tremblay et al., 1999).

The precise role of COX-2 in carcinogenesis is not known, however various potential mechanisms have been proposed (Hussain et al., 2003). Evidence showing reduced angiogenesis in tumours after treatment with specific COX-2 inhibitors suggest that COX-2 may favour tumour growth by inducing endothelial cell proliferation and migration, thereby promoting angiogenesis in rapidly growing solid tumours (Zha et al., 2004). Another

proposed mechanism for the role of COX-2 in cancer progression is resistance to apoptosis (Kirschenbaum et al., 2001; Johnson et al., 2001). However, these hypotheses are derived from research on the effects of COX-2 inhibitors. Since these compounds may have COX-2-independent effects, it is not clear whether the inhibition of COX-2 alone, or other effects are responsible for the apoptosis induced by these products (Zha et al., 2004).
COX-2 is expressed in both human and canine prostate carcinoma (PCA). In two immunohistochemistry studies of canine PCA, COX-2 expression was found in 75% and 88.2% of the tumors, respectively (Sorenmo et al., 2004; Tremblay et al., 1999), whereas it was not expressed in normal prostatic tissue. These findings suggest that COX-2 may play a role in the pathogenesis of PCA in dogs. In human PCA, COX-2 expression has been reported to be significantly higher in poorly differentiated compared to well differentiated tumours (Madaan et al., 2000), and to correlate with local chronic inflammation (Wang et al., 2005). Canine PCA is histologically a heterogeneous tumour that may present several patterns of histological differentiation(Cornell et al., 2000; Leav & Ling, 1968; Young et al., 2000) and a possible correlation between COX-2 expression and histological classification of the tumours or presence of inflammation has not yet been examined.
Little is known about the mechanisms regulating the expression of COX-2 in tumor cells. However, it has previously been shown that the expression of COX-2 can be up-regulated by oncogenes, growth factors, cytokines, endotoxins and phorbol esters (Chen et al., 2005; Boutemmine et al., 2002; Arias-Negrete et al., 1995; Inoue et al., 1995).

Exogenous risk factors (human PCA)
Many exogenous risk factors have been studied and their possible aetiological role in human PCA examined. The only two that seemed to be associated with a higher risk of PCA were consumption of animal fat and exposure to pesticides and/or farming (Bostwick et al., 2004).

Clinical features and diagnosis
Clinical signs
The frequency of clinical signs observed in dogs with PCA varies from report to report (Bell et al., 1991; Cooley & Waters, 2001; Cornell et al., 2000; Krawiec & Heflin, 1992) and are not specific (Teske & Nickel, 1996). The most common signs are straining to defecate (dyschezia), problems with urination (stranguria, haematuria) and systemic signs (weight loss, anorexia). Occasionally, signs of hind limb lameness or neurological deficits may be seen in cases where metastasis to the lumbar spine, pelvis or femur has occurred. Prostatamegaly is found on physical examination. Prostate enlargement is more often asymmetrical than symmetrical (Bell et al., 1991).

Laboratory and imaging findings
No haematologic or serum chemistry findings are specific for PCA in dogs. The most frequently encountered change is leucocytosis (Bell et al., 1991). Canine prostate-specific arginine esterase (CPSE) is a secretory product of the canine prostate and a known marker of

prostate secretion in the dog. However, unlike PSA in people, its potential diagnostic use is limited to non-neoplastic diseases of the prostate (Gobello et al., 2002).
The most common radiographic and ultrasonographic findings (**Figure 3**) are enlargement of the prostate and the presence of intraprostatic mineralization (Bell et al., 1991; Feeney et al., 1987a; Feeney et al., 1987b).

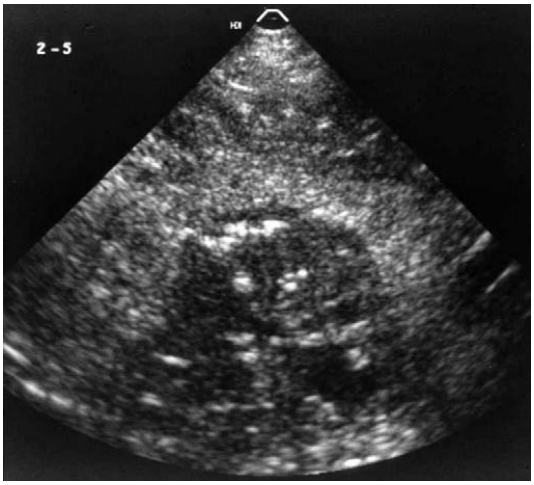

Figure 3. Ultrasound examination of prostate carcinoma in a dog. Note the hyperechoic foci indicating mineralization within the prostatic parenchyma.

The presence of the above mentioned clinical and diagnostic imaging findings in an older castrated male dog constitutes a very strong suspicion of PCA. However, definitive diagnosis is obtained by cytological examination of fine needle aspiration biopsies of the prostate, if necessary in combination with a catheter aspiration biopsy (Teske & Nickel, 1996). Biopsies are best taken under ultrasound guidance. Typical cytological findings (**Figure 4**) include irregular shape and size of prostatic cells, presence of multiple nucleoli and a strong basophilic staining of the cytoplasm (Teske & Nickel, 1996).

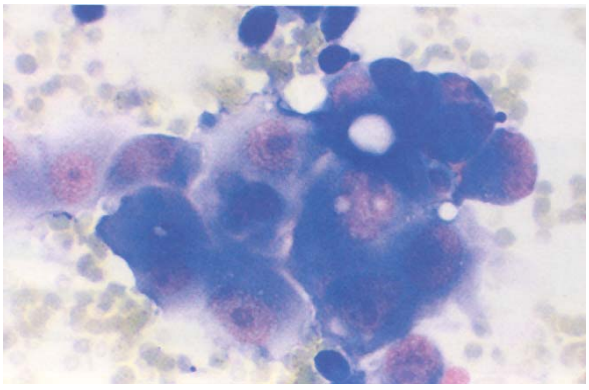

Figure 4. Fine needle aspirate of prostate carcinoma in a dog: note the irregular shape of the cells, the strong basophilic staining of the cytoplasm and the presence of multiple nucleoli.

In humans, the diagnosis of the disease is made by detecting elevated serum levels of prostate specific antigen (PSA), by digital rectal examination (DRE), by transrectal ultrasound exam (TRUS) and histologically grading the tumour tissue subsequently to prostate biopsy (Alivizatos et al., 1996; Donovan Jr., 1994). PSA is normally secreted into the glandular lumen of the prostate by columnar epithelial cells, but leaks into the serum in PCA because of damage to the basement membrane (Schalken, 2004). Many patients with slightly elevated serum PSA values undergo unnecessary biopsy procedures (Alivizatos et al., 1996). This has caused many authors to advocate differentiation between the levels of free PSA, complexed PSA and total PSA in order to enhance the specificity of PSA testing when determining which patients should undergo prostate biopsy (Alivizatos et al., 1996; Jung et al., 2001; Okegawa et al., 2003).
The histological grading of human prostate carcinoma is known as the Gleason grading scheme (Gleason & Mellinger, 1974). The Gleason score of the tumour is used as a predictive factor for the outcome of the disease. Beside the Gleason score prostate tumours can also be staged using the TNM system shown in **Table 1** (Hoedemaeker et al., 2000).

Table 1. TNM staging of prostate cancer (Hoedemaeker et al., 2000)

T Stages
T followed by numbers 0 through 4 refers to the size and extent of the tumor itself.

Stage		**Description**
0	**T0**	No evidence of tumor
1	**T1**	The tumor cannot be felt or seen using imaging techniques. Incidental finding.
	T1a.	Cancer cells are incidentally found in 5% or less of tissue samples from transurethral prostate resection.
	T1b.	Cancer cells found in more than 5% of samples.
	T1c.	Cancer cells identified by needle biopsy, which is performed because of high PSA levels.
2	**T2**	The cancer is confined to the prostate but can be felt as a small well-defined nodule.
	T2a.	Tumor in one side of the prostate.
	T2b.	Tumors in both lobes of the prostate.
3	**T3a**	The tumor extends through the prostate capsule
	T3b	Seminal vesicle invasion on one or both sides
4	**T4**	The tumor is fixed to or invades adjacent structures.

N Stages
N followed by 0 to 3 refers to whether the cancer has reached the regional lymph nodes, which are located next to the prostate in the pelvic region.

Stage	**Description**
N0	Regional lymph nodes are still cancer-free.
N1	A small tumor is in a single pelvic node.
N2	A medium-size tumor is in one node or small tumors are in several nodes.
N3	A large tumor is in one or more nodes.

M Stages
M stages refer to metastasis (tumors developing outside the prostate).

Stage	Description
M0	Metastasis has not occurred (cancer has not spread beyond the regional lymph nodes).
M1a	Cancer has spread to lymph nodes beyond the regional lymph nodes.
M1b	Cancer has invaded the bones.
M1c	Cancer has spread to other sites.

Treatment
Most dogs diagnosed with PCA are not treated, either because of the presence of metastases, or because of the poor prognosis. Little data is available on life expectancy after diagnosis if no treatment is attempted. One study reports that 58 of 72 dogs were euthanised at the time of diagnosis and that the median survival time for the remaining 12 dogs was 30 days (Cornell et al., 2000). Whether treatment was attempted in these 12 dogs is not mentioned.

Endocrine therapy
The principle of endocrine therapy is the elimination of prostate stimulation by androgens. Castration in dogs does not have a sparing affect on the development of PCA (Obradovich et al., 1987) and in fact PCA occurs more often in castrated dogs than intact males (Teske et al., 2002). In intact male dogs with PCA, castration does not have have a favourable influence on outcome either. Therefore endocrine therapy plays no role in the treatment of PCA in dogs. In humans, endocrine therapy is the mainstay for the management of advanced PCA that cannot be cured with radical local therapy, either surgery or radiation therapy (Damber, 2005). Androgen withdrawal can be achieved by surgical castration, administration of oestrogens, GnRH antagonists or anti-androgens. The more recently developed non-steroidal antiandrogens such as bicalutamide and hydroxyflutamide have fewer side effects than castration and have become first choice compounds for the endocrine reatment of PCA (Damber, 2005; Lam et al., 2006). However, in most cases the tumour progresses to an androgen-independent state (Feldman & Feldman, 2001). Resistance to treatment occurs an average of 18 to 24 months after the beginning of hormone ablation (Lam et al., 2006). Androgen-independent prostate cancer tends to progress and metastasise and has a median survival of 12 to 18 months (Lam et al., 2006). Other treatment options for patients with progression of prostate cancer and low levels of androgens include (in no particular order) chemotherapy, external beam radiation therapy, brachytherapy, surgery, minimal invasive techniques and biomolecular therapies.

Surgery
Treatment with curative intent is rendered difficult by the fact that radical prostatectomy in dogs with prostatic disease is associated with a very high incidence of postoperative urinary incontinence (Basinger & Rawlings, 1987; Basinger et al., 1987; Goldsmid & Bellenger, 1991). Interestingly, radical prostatectomy can be performed in normal dogs without causing

incontinence (Basinger et al., 1989; Price et al., 1996). It therefore seems that urethral sphincter malfunction is more likely due to primary prostatic disease than to the surgical procedure. Because of these complications, techniques for partial removal of the prostate have been described. These include partial prostatectomy using a Nd:YAG laser (Hardie et al., 1990) and intracapsular subtotal prostatectomy using electrocoagulation (Harari & Dupuis, 1995) or an ultrasonic aspirator (Rawlings et al., 1994; Rawlings et al., 1997). However these techniques have been used for the management of non-neoplastic prostate diseases in dogs (e.g. abcesses and cysts) and neither of them have been used to treat PCA.
Reports of surgical treatment of PCA in dogs are limited to a few cases. Surgical placement of a retained urethral catheter in three dogs with PCA and stranguria enabled the dogs to survive 3 to 5 months after surgery (Mann et al., 1992). In a recent study, 3 male dogs with prostatic neoplasia (prostatic transitional cell carcinoma in 2 cases and undifferentiated carcinoma in one case) were treated with transurethral resection using an electrosurgical loop (combined with intraoperative radiation therapy in 2 of those 3 dogs) (Liptak et al., 2004). Survival times were 32, 74 and 264 days.
In humans, radical prostatectomy is a potential curative treatment for prostate cancer, provided the disease is localized to the prostate (T1-T3a, N0, M0, see **Table 1**) (Damber & Khatami, 2005). Metastases-free survival 15 years after surgery are reported to be between 76% (Zincke et al., 1994) and 82% (Han et al., 2001). The procedure can be performed via a retropubic or a perineal approach, or by laparoscopy (Damber & Khatami, 2005). Retropubic radical prostatectomy is considered the gold standard surgical technique and is one of the most commonly performed urologic surgical procedures performed. The procedure has been perfected in the last two decades, so that neurovascular bundles responsible for urethral sphincter function can be spared, thereby greatly reducing the incidence of postoperative complications such as urinary incontinence and erectile dysfunction (Damber & Khatami, 2005).

Radiotherapy
There are no reports on radiotherapy alone in dogs. Radiotherapy has been attempted in combination with surgery. In one study 10 dogs underwent intraoperative orthovoltage radiation (Turrel, 1987). The mean survival time was 114 days. In another study external beam radiation therapy was combined with administration of ketoconazole. Two dogs treated with this technique were reported to survive for 12 weeks and 4 months, respectively (Bell et al., 1991).
In humans, radiotherapy is most often performed as brachytherapy, a technique involving the placement into the prostate of multiple small seeds of radioactive isotopes (Ciezki, 2005). Expectations for cure are between 80% and 90% (Ciezki, 2005).

Chemotherapy
No effective chemotherapeutic protocol is available for the treatment of PCA in dogs. In humans, most chemotherapeutic agents have been shown to palliate symptoms, but will not prolong survival of patients. The only exception is docetaxel (Silvestris et al., 2005).

COX-2 inhibitors

As mentioned above, COX-2 is expressed in various types of canine cancer including PCA, and is thought to play a role in carcinogenesis. This is supported by evidence that treatment with COX-2 inhibitors may be beneficial in the management of certain tumours in dogs. In particular piroxicam has been shown to effectively inhibit transitional cell carcinoma (TCC) in dogs (Knapp et al., 1992; Knapp et al., 1994; Henry et al., 2003). Although it has been shown that COX-2 is expressed in canine PCA, the effect of NSAIDs on the clinical outcome of dogs with PCA has not been extensively investigated. Preliminary findings in dogs with PCA (Sorenmo et al., 2003) and in a mouse prostate carcinoma model (Gupta et al., 2004) suggest that COX-2 inhibitors may play a significant role in the management of PCA in dogs.

Photodynamic therapy

Photodynamic therapy involves the use of a photosensitizing drug and subsequent delivery of light in the presence of oxygen. The resulting photochemical reaction releases various oxygen radicals that are highly toxic (Ahmed et al., 2005; Peng et al., 1997).

Treatment of PCA with transurethral photodynamic therapy allowed the only dog reported to survive nearly 9 months after treatment (Lucroy et al., 2003).

A number of different photosensitizers have been tested in healthy dogs. These include dihematoporphyrin ester/ether (Photofrin), meso-tetra-(m-hydroxyphenyl) chlorin (mTHPC) (Chang et al., 1996), aluminium disulfonated phthalocyanine (AlS_2Pc) (Chang et al., 1997), 5-aminolevulinic acid (ALA) (Chang et al., 1997), tin ethyl etiopurpurin ($SnET_2$) (Selman and Keck, 1994; Selman et al., 2001) and Motexafin Lutetium (Lu-Tex) (Hsi et al., 2001). Photofrin is a so-called first generation photosensitizer and has been associated with significant skin photosensitization for several weeks after administration. The other photosensitizers tested in the dog are second-generation photosensitizers with reduced skin photosensitization because of a faster elimination and are therefore more appropriate to use for PDT of prostate carcinoma in dogs. Dogs receiving AlS_2Pc and ALA were kept in a dimly lit area and for the other studies the dogs were kept in normally illuminated cages but not exposed to direct sun light.

The administration of light to the prostate after intravenous injection of the photosensitizer can be performed in a number of ways: interstitial PDT is the administration of light to the parenchyma of the prostate through several light fibres inserted into the prostate from the perineum. Transurethral PDT is the administration of light through a light cable inserted into the urethra like a urinary catheter. This technique is technically easy but its efficiency is limited by the depth of light penetration into prostate parenchyma (usually less than 20 mm) (Selman et al., 2001).

The largest area of necrosis was obtained using $SnET_2$ with lesions of up to 26 mm diameter reported after interstitial PDT and 10 mm necrosis around the urethral wall reported after transurethral PDT (Selman et al., 2001). With the other photosensitizers, the lesions seen were between 10 and 20 mm in diameter. After transurethral PDT the urethral mucosa is destroyed but regenerates within 3 weeks of treatment (Selman et al., 2001).

In humans, PDT is emerging as a promising minimally-invasive treatment modality for PCA (Moore et al., 2005). However the ideal photosensitiser and light delivery device has not yet been found and investigations are ongoing to evaluate the benefits of PDT for the management of early PCA in clinical practice (Moore et al., 2005).

Minimally invasive treatment techniques

With the development of more sensitive diagnostic techniques, particularly the use of PSA as a clinical marker for prostate cancer, the condition is more frequently being diagnosed earlier, at a stage where the disease is still localized to the prostate, even though there is a risk of putting a number patients through unnecessary biopsy procedures, because of the lack of specificity of PSA measurements. More recently minimally invasive techniques for the management of such localized prostate cancer have been developed. These include cryotherapy, high-intensity focused ultrasound (HIFU), microwave and radiofrequency interstitial tumour ablation (RITA) (Ahmed et al., 2005).

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CHAPTER 2

AIMS AND SCOPE OF THE THESIS

Animal models have always been invaluable aids for studying human disease. Due to variations in the anatomy of the male urogenital system, the availability of animal models for the study of prostatic diseases is rather limited. The dog and the chimpanzee have been used as models for benign prostatic hyperplasia (BPH) in humans (Mahapokai et al., 2000; Steiner et al., 1999) as both these species develop spontaneous BPH. In addition BPH can be hormonally induced in the dog, thus creating a good model for the disease in humans (Mahapokai et al., 2000).

Although prostate-specific antigen-related genes have been found in several species of non-human primates (Karr et al., 1995), indicating some degree of physiological similarity with the human prostate, there are no reports of spontaneous prostate carcinoma in these primates. The fact that prostate carcinoma (PCA) occurs spontaneously in dogs, combined with practical considerations have made the dog the preferred model for studying prostate cancer (Waters and Bostwick, 1997a). Indeed PCA in dogs shares many features of the disease in humans. In both species, prostate cancer is diagnosed in older individuals, and the pattern of metastases spread is the same with the local lymph nodes, the lungs and the skeleton being the primary sites of metastasis (Cooley and Waters, 2001). PCA is frequently diagnosed in humans before it starts causing symptoms either by transrectal ultrasound (TRUS) or prostate-specific serum antigen (PSA) monitoring. Sometimes it is diagnosed as an incidental finding in men treated for presumed benign prostatic hyperplasia, when prostatic tissue is removed to relieve a bladder outlet obstruction. In these cases, the neoplasia is usually limited to a portion of the prostate. In more advanced stages, PCA in humans causes urinary obstruction and/or urinary infection and, in the case of metastatic disease, weight loss, bone pain and neurological deficits. In dogs, there is no screening test and PCA is unfortunately commonly diagnosed late in the course of the disease, when neoplastic tissue has invaded the entire gland and causes urinary obstruction or when prostatamegaly causes straining to defaecate. Weight loss is also a common feature of PCA in dogs.

Additional similarities between human and canine PCA include the presence of the preneoplastic lesion prostatic intraepithelial neoplasia (PIN) characterised by groups of cells with papillary infoldings and nuclear hyperchromatism and pleomorphism (Aquilina et al., 1998; Waters and Bostwick, 1997b). In humans the risk of developing PCA has been reported to vary between different ethnic groups (Bostwick et al., 2004; Jemal et al., 2005; Winters et al., 2001) and African-American men have the highest incidence of PCA in the world, whereas Japanese men are reported to have a relatively low risk of developing PCA. Epidemiologic studies in dogs have also shown breed variations in the risk of PCA and the Bouvier des Flandres has been found to have a significantly higher risk of developing PCA compared to the reference population (Teske et al., 2002).

However there are also essential differences between canine and human PCA. Prostatic carcinoma in humans is a common neoplasm and is initially a hormone-dependent disease responding well to hormone ablation therapy (Huggins and Hodges, 2002). In dogs, PCA is rare and occurs more frequently in castrated males than in intact males (Bell et al., 1991; Bryan et al., 2007; Obradovich et al., 1987; Teske et al., 2002), indicating that, contrary to humans, the presence of androgens is not required for disease progression.

Current research on the pathogenesis of prostate cancer focuses on several aspects including the role of androgens, the expression of the androgen receptor, the sequence of the androgen

receptor gene, the regulation of proliferation and apoptosis and the role of cyclooxygenase-2 (COX-2).
In humans, the androgen-androgen receptor axis is believed to play a major role in prostate cancer. The function of the androgen receptor in the normal prostate epithelium is to induce differentiation and inhibit growth signals. However, in prostate cancer, AR stimulation is believed to induce proliferation of the cancer cells. The human AR-gene contains a polymorphic CAG repeat which has been linked to prostate cancer risk: humans with shorter AR-CAG repeat lengths are at greater risk of developing prostate cancer than are those with longer variants. Moreover, ARs with shorter CAG repeats have been found to be transcriptionally more active. The role of the androgen-androgen receptor axis in canine prostate cancer is not yet clear.
In humans, differences in serum concentrations of androgens may explain the racial differences in prostate cancer risk as African-American men have been found to have higher serum concentrations of testosterone and sex-hormone binding-globulin (SHBG) (Winters et al., 2001). In dogs, it is not known whether the serum androgen concentration varies between breeds.
Cyclooxygenase-2 (COX-2) expression has been documented in human and canine prostate carcinoma (PCA), however little is known about the mechanisms regulating COX-2 expression in neoplastic tissue. Canine PCA is a histologically heterogeneous tumor, sometimes including inflammatory infiltrations and it is unknown whether COX-2 expression in canine PCA is related to the histological type of the tumor and/or to the presence of inflammation.
This thesis was conducted with two aims: the first aim of this thesis is to obtain an insight into the pathogenesis of canine prostate carcinoma by (1) characterising the pattern of COX-2 expression in canine prostate carcinoma, (2) investigating the variations in the androgen receptor gene in dogs with prostate carcinoma, and (3) examining the serum level of androgens in the Bouvier des Flandres, a breed of dog found to have a higher probability of developing prostate carcinoma than the reference population.
Although many different treatment modalities have been attempted, there is currently still no effective therapy for PCA in dogs and most dogs are euthanised at the time of diagnosis. The second aim of this thesis is therefore to develop and evaluate therapeutic tools for canine prostate carcinoma.

In the General Introduction (**chapter 1**), an overview of the anatomy and physiology of the prostate as well as of the pathogenesis and clinicopathological features of prostate cancer in humans and dogs was presented, with particular emphasis on similarities and differences between the species, limiting the use of the dog as a model for human PCA.
Cyclooxygenase-2 (COX-2), the inducible form of the enzyme has been found to be overexpressed in various types of cancer including prostate carcinoma. The expression of COX-2 was studied in canine prostate carcinoma tissue as well as in cell cultures of both cells from normal canine prostate and cells from canine prostate carcinoma (**chapter 3**).
In humans, the risk of developing prostate carcinoma has been related to variations in the sequence of the androgen receptor gene. The relationship between variations in the androgen

receptor gene sequence and prostate carcinogenesis in the dog was investigated using DNA from healthy dogs and dogs with PCA (**chapter 4**).
Despite the higher incidence of prostate carcinoma in castrated dogs than in intact male dogs, it cannot be ruled out that androgens play a role in the pathogenesis of canine prostate carcinoma, nevertheless. The role of serum androgen levels in the pathogenesis of prostate carcinoma was investigated as a possible explanation for the apparent predisposition of the Bouvier des Flandres dog breed (**chapter 5**).
Radical prostatectomy in dogs is followed by a very high incidence of postoperative urinary incontinence. Therefore surgical management of prostate carcinoma requires preserving the dorsal aspect of the prostatic capsule in order to maintain neurovascular supply and urethral sphincter function. Dissection through prostate parenchyma requires precision and good haemostasis in order to achieve maximal removal of neoplastic tissue without damaging vital structures. A partial subcapsular prostatectomy technique using a Nd:YAG surgical laser was therefore developed. The safety of laser dissection in the vicinity of vital structures such as the urethra and neurovascular supply to the dorsal part of the prostate was examined first *in vitro* (**chapter 6**). Then the technique was tested in 4 experimental dogs before being used in patients. By definition, partial prostatectomy cannot achieve complete removal of neoplastic tissue and additional therapy is required to control the growth of remaining carcinoma cells. In the first patients treated partial surgical removal of the prostate was combined with local injections of interleukin-2 into the prostate and with systemic treatment with the COX-2 inhibitor meloxicam (**chapter 7**).
Photodynamic therapy (PDT) is a relatively recent cancer treatment modality involving the use of a photosensitizer and subsequent delivery of light to the neoplastic tissue. In patients with PCA, photodynamic therapy including local application of a photosensitizer to the prostate and intraoperative treatment with red light was evaluated instead of interleukin-2 for the local control of tumour cells remaining after laser dissection (**chapter 8**).

The findings of all subsections are summarised and discussed in the general discussion (**chapter 9**). The thesis is concluded with a summary (**chapter 10**).

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CHAPTER 3

Regulation of COX-2 expression in canine prostate carcinoma: increased COX-2 expression is not related to inflammation

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Abstract

Background: Cyclooxygenase-2 (COX-2) expression has been documented in human and canine prostate carcinoma (PCA). Canine PCA is a histologically heterogeneous tumor, sometimes including inflammatory infiltrates. However, it is unknown whether COX-2 expression in canine PCA is related to the histological type of tumor, to the presence of inflammation or to both. Moreover, little is known about the mechanisms regulating COX-2 expression in neoplastic tissue.
Hypothesis: COX-2 expression is related to the presence of inflammation in canine PCA and correlates with the degree of tumor differentiation.
Methods: The expression of COX-2 was examined in 28 cases of canine PCA using immunohistochemistry. In addition, a neoplastic and a non-neoplastic canine prostatic cell line were used to investigate the effects of interleukin-6, tumor necrosis factor-α, phorbol 12-myristate 13-acetate, epithelial growth factor and specific signal transduction pathway inhibitors on COX-2 expression.
Results: Twenty-four of the 28 prostate tumors showed COX-2 expression. The presence of inflammatory infiltrates in tumor tissue was associated with lower COX-2 expression scores. In vitro, TNF-α, IL-6 and EGF increased COX-2 expression in non-neoplastic cells but not in PCA cells, where baseline expression was high. COX-2 expression in PCA cells could be suppressed using specific PI3K, PKC or ERK/MAPK inhibitors.
Conclusions and clinical importance: COX-2 is expressed in canine PCA, however expression is not related to the presence of inflammatory infiltrates. This conclusion is further supported by the finding that the cytokines TNF-α and IL-6 and their involved signaling pathways do not stimulate COX-2 expression in malignant canine prostate cells.

Introduction

The enzyme prostaglandin-endoperoxide synthase, also referred to as cyclooxygenase (COX), exists in 2 main isoforms. COX-1 is constitutively expressed in many cell types[1], whereas COX-2 is the inducible form of the enzyme and is only expressed in cells involved in various inflammatory and neoplastic diseases[1-3]. The COX-2 enzyme itself and its RNA expression have been detected in several forms of human and canine cancer[3]. In the dog, these include oral squamous cell carcinoma, mammary tumors, transitional cell carcinoma of the urinary bladder and prostatic carcinoma[4-9]. There is a growing body of evidence showing that COX-2 and its product prostaglandin E_2 (PGE_2) promote tumor development by a variety of mechanisms, such as an increase in proliferation, a decrease in apoptosis and an induction of angiogenesis, possibly by generating free radicals and carcinogens[1,10].

COX-2 is expressed in both human and canine prostate carcinoma (PCA). In 2 immunohistochemistry studies of canine PCA, COX-2 expression was found in 75% and 88.2% of the tumors, respectively [4,9] whereas it was not expressed in normal prostatic tissue. These findings suggest that COX-2 may play a role in the pathogenesis of PCA in dogs. In human PCA, COX-2 expression has been reported to be significantly higher in poorly-differentiated as compared to well-differentiated tumours[11], and to correlate with local chronic inflammation[12]. Canine PCA histologically is a heterogeneous tumor that may present several patterns of histological differentiation[13-15], and a possible correlation between COX-2 expression and histological classification of the tumors or presence of inflammation has not yet been examined.

Little is known about the mechanisms regulating the expression of COX-2 in tumor cells. However, it previously has been shown that the expression of COX-2 can be up-regulated by oncogenes, growth factors, cytokines, endotoxins and phorbol esters[16-19].

The objectives of this study were to examine whether there is a relationship between the expression of COX-2 and either the histological morphology of the tumors or the presence of inflammation in canine PCA, as well as to gain insight into the mechanisms responsible for COX-2 expression in canine PCA. For this, COX-2 expression in naturally-occurring canine PCA was examined by immunohistochemistry. Furthermore, the ability of the tumor promoter phorbol 12-myristate 13-acetate (PMA), the cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) as well as epithelial growth factor (EGF) to induce up-regulation of COX-2 expression was investigated in normal and neoplastic canine prostatic cell lines. In order to understand which signaling pathways are used for this up-regulation, the cells also were incubated with 3 inhibitors of the various signaling pathways: PD98059 (inhibitor of extracellular signal related kinase [ERK/MAPK])[20,21], GF109203X (inhibitor of protein kinase C [PKC])[22], and LY294002 (inhibitor of phosphatidyl inositol-3 kinase [PI3K]).

Materials and methods

Tissue samples
Prostate tissue from 28 dogs with prostatic carcinoma was used for immunohistochemistry staining of COX-2. The dogs included 21 castrated and 7 intact males with prostatic carcinoma. Tissue was obtained either during surgery or during necropsy. Tissue samples were fixed in neutral buffered formalin and embedded in paraffin using standard protocols.

Immunohistochemistry staining
Five micrometer paraffin-embedded tissue sections were cut and mounted on Superfrost® slides[a]. Subsequently, the slides were dewaxed in xylene[b] and rehydrated in descending concentrations of alcohol[b] (100%, 96%, 70%). After rinsing the slides in distilled water, the tissue was pre-treated by boiling the slides in 0.01 M citrate[b] buffer, pH 6, for 10 min in a microwave oven. The slides were left to cool for 30 min and rinsed in phosphate buffered saline (PBS) before the endogenous peroxidases were inhibited by immersing the tissue in 0.3% hydrogen peroxide[b] in methanol[b] for 30 min at room temperature. The slides again were rinsed in PBS, and non-specific antibody binding was blocked by incubating the sections with normal horse serum (NHS) diluted 1:10 in PBS for 30 min at room temperature. Next, the tissue sections were incubated overnight at 4°C with the primary antibody (monoclonal mouse anti-COX-2[c]) diluted 1:50 in PBS. After rinsing with PBS, the sections were treated for 45 min at room temperature with the secondary antibody (horse-anti-mouse biotinylated IgG[d]), at a concentration of 1:125 in PBS. Visualization of the immune reaction then was performed using an ABC/peroxidase kit[d], and diaminobenzidine[e] as a substrate (0.05% solution in 0.05 M Tris-HCl, pH 7.8, with 0.003% H_2O_2). After a final rinse in distilled water, the sections were briefly counterstained (15 sec in hematoxylin) and dehydrated (successive concentrations of 70%, 96%, 100% ethanol and finally xylene). The specificity of the primary antibody was tested by performing a Western blot on canine PCA cells and obtaining a single stained band just below the 75 kDa marker, which corresponds with the molecular weight of COX-2 (72 kDa). Sections of normal canine bladder were used as negative control and sections of canine transitional carcinoma of the bladder were used as positive control.

Classification of histological subtypes
The tumors were classified according to criteria described by Leav and Ling[15]. The different histological subtypes described and observed were ductal, micropapillary, sarcomatoid, small acinar and solid.

Immunohistochemistry scoring
The slides were evaluated with regard to number of stained cells, intensity of the staining and presence of inflammation. The number of stained tumor cells was estimated and scored as 0

[a] Menzel Glaser, Braunschweig, Germany
[b] Merck, Darmstadt, Germany
[c] Zymed Laboratories Inc., San Francisco, CA, USA
[d] Vector Laboratories, Burlingame, CA, USA
[e] Sigma-Aldrich Chemie GmbH, Steinheim, Germany

(0% positive cells), 1 (>0-20% positive cells), 2 (>20-40% positive cells), 3 (>40-60% positive cells), 4 (>60-80% positive cells) and 5 (>80-100% positive cells). Intensity was scored as 0 (no staining), 1 (mild staining), 2 (intermediate staining) and 3 (strong staining). Inflammation was scored as 0 (absent), 1 (mild), 2 (intermediate) and 3 (marked). In addition, a staining index was calculated by multiplying the score for the number of positive cells (0 to 5) by the score for staining intensity (0 to 3), as previously described [4,23]. Different areas of the same slide were scored separately if they differed in histological subtype or degree of inflammation.

Cell culture

The non-malignant canine prostate cell line CAPE[24,25] was a generous gift of Dr. Tom Rosol (Columbus, Ohio) and the malignant cell line ACE was obtained from Dr. Helen Jones (Cardiff, UK). Both cell lines were maintained at 37 °C in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium[f] (DMEM) with the addition of 5% fetal calf serum[f] (FCS).

Twenty four h before the experiments, the culture medium was replaced with DMEM without FCS. Then, the cells were incubated for 24 h with putative activators of COX-2 (PMA[g], 10 ng/ml; IL-6[g], 10 ng/ml; EGF[g], 10 ng/ml; TNFα[g], 50 ng/ml) either alone or in combination with several specific signal transduction pathway inhibitors (PD98059[h], GF109203X[i] and LY294002[h], at a concentration of 10 μM). After 24 h of incubation, the cells were washed with Hanks' balanced salt solution[f] (HBSS) and harvested by scraping them from the culture flask and suspending them in approximately 0.5 ml HBSS. The cells were centrifuged and stored without buffer at –80 °C until further processing. All experiments were performed in duplicate.

Measurement of protein content

Cells were lysed with lysis buffer containing 1% igepal[e], 0.5% sodium deoxycholate[e] and 0.1% sodium dodecyl sulfate[b] (SDS) in phosphate buffered saline (PBS). Samples were incubated for 30 min on ice, and centrifuged at 4 °C for 3 min at 1000 rpm. The supernatant was carefully removed and stored at –20 °C until further use. The protein content of the samples was determined using a protein assay kit[j] and serial dilutions of bovine serum albumin[e] (BSA) as a standard.

Western blot

Five μg protein from each sample was suspended in a buffer containing 20% glycerol[b], 2.5% SDS and 0.5% brome phenol blue[b] in 0.125 M Tris-HCl[b] buffer at pH 6.8, and separated by electrophoresis on ready-made 10% Tris-HCl gels[k]. Then, the gels were equilibrated for 30

[f] Gibco Invitrogen, Paisley, Scotland, UK
[g] Sigma chemical Company, St Louis MO, USA
[h] Calbiochem, La Jolla, CA, USA
[i] Biomol, Plymouth Meeting, PA, USA
[j] *DC* Protein Assay, BioRad Laboratories, Hercules, CA, USA
[k] Tris-HCL gels, BioRad

min in transfer buffer (25 mM Tris, 192 mM glycine[e], 20% methanol[l]), before the protein was transferred from the gels to nitrocellulose membranes[m]. These blots then were washed in Tris-buffered saline (TBS) and blocked for 1 h in 3% non-fat dry milk[b] in TBS at room temperature. They then were incubated for 1 h at room temperature with the primary antibody (Mouse anti-COX-2) at a concentration of 1:1000 in TBS containing 0.1% Tween-20[n] (TBST). The blots were washed in TBST and incubated for 1 h at room temperature with the secondary horseradish peroxidase (HRP)-linked anti-mouse antibody[o] at a concentration of 1:20,000. The blots were washed in TBST and TBS, and stained using a chemiluminescent substrate kit[p] before being exposed to an x-ray film for 30 sec.

Statistical methods

Where appropriate, differences in immunohistochemical scoring were analyzed using the non-parametric Mann-Whitney test. Statistical significance was accepted for P values < 0.05.

Results

COX-2 expression in prostate carcinoma tissue

Nineteen of the 28 dogs examined (68%) had more than 1 histological subtype identified in the sections investigated. A total of 50 distinct tumor areas were identified in the 28 prostate glands examined (i.e. areas with different histological subtypes or different degrees of inflammation). The distribution of histological types between intact and castrated dogs is summarized in Table 1. In both castrated and intact dogs, the micropapillary and solid tumor subtypes were the most frequently seen. Both subtypes together represented 88% (44/50) of the different tumor areas (micropapillary 40% [20/50] and solid 48% [24/50]). The ductal, small acinar and sarcomatoid subtypes were seen in 6% (3/50), 4% (2/50) and 2% (1/50) of the cases, respectively. The micropapillary type was seen in 34% (13/38) of the cases in castrated dogs and in 58% (7/12) of the cases in intact dogs. The solid type was seen in 55% (21/38) of the cases in castrated males and in 25% (3/12) of the cases in intact males. These differences between castrated and intact males were not statistically significant.

[l] Labscan Ltd., Dublin, Ireland
[m] Hybond C, Amersham Biosciences, Amersham, UK
[n] Boom, Meppel, The Netherlands
[o] Anti-mouse IgG, R&D Systems, Minneapolis, MN, USA
[p] Immune-star HRP kit, BioRad Laboratories, Hercules, CA, USA

Table 1. Distribution of histological types among castrated and intact males

		Castrated	Intact	Total
No. of dogs		21	7	28
No. of dogs with one hstological type		7	2	9
No. of dogs with several histological types		14	5	19
Total no of histological distinct areas		38	12	50
Of which:	Solid	21	3	24
	Micropapillar	13	7	20
	Ductal	2	1	3
	Small acinar	1	1	2
	Sarcomatoid	1	0	1

The immunohistochemistry scoring is summarized in Table 2. There was no statistically significant difference in the mean degree of inflammation between the micropapillary and solid tumor subtypes. Inflammation was present as frequently in tumors of castrated dogs as in those of intact dogs, however the degree of inflammation in intact dogs was significantly lower than that in castrated dogs (P=0.021).

Table 2. Summary of the immunohistochemistry scoring (mean ± SD)

	No of positive cells	Intensity of staining	Staining index	Degree of inflammation
Castrated males	2.82 ± 1.75	2.21 ± 0.95	6.12 ± 4.11	1.73 ± 1.11
Intact males	3.60 ± 1.71	2.50 ± 0.71	9.20 ± 4.94	0.80 ± 0.79
(P value)[1]	(P = 0.17)	(P = 0.43)	(P = 0.07)	(P = 0.02)
Solid type[2]	3.00 ± 1.83	2.25 ± 0.91	7.15 ± 4.93	1.65 ± 1.18
Micropapilary type[2]	3.00 ± 1.72	2.29 ± 0.91	6.54± 4.08	1.42 ± 1.06
(P value)[1]	(P = 0.92)	(P = 0.85)	(P = 0.77)	(P = 0.46)
No inflammation	4.36 ± 0.81	2.45 ± 0.69	10.82 ± 4.02	n/a[3]
Inflammation score 1, 2 or 3	2.55 ± 1.75	2.21 ± 0.96	5.48 ± 3.77	n/a[3]
(P value)[1]	(P = 0.004)	(P = 0.58)	(P = 0.001)	n/a[3]

[1]Using the non-parametric Mann-Whitney test (statistical significance accepted for $P \leq 0.05$
[2]Other types not included in the statistical evaluation because of the small number of cases
[3]Not applicable

Positive COX-2 staining (see Figure 1) typically was seen as granules in the cytoplasm, often with increased density around the nuclei of the cells. There was no staining of stromal tissue. Three of the 28 tumors (10.7%) stained negative for COX-2. Three of the 4 negative tumors were from castrated dogs and 1 from an intact male. Two were ductal, 1 micropapillary and 1 solid. There was no significant difference in staining index among histological subtypes (P=0.767). The median staining index was 7 for the micropapillary subtype, and 5.5 for the solid subtype. Although there was no significant difference in the number of positively-stained cells or the intensity of staining between castrated and intact dogs, there was a lower but non-significant staining index in castrated dogs compared to intact dogs (P=0.07), with a median staining index of 5 for castrated dogs and 8 for intact dogs.

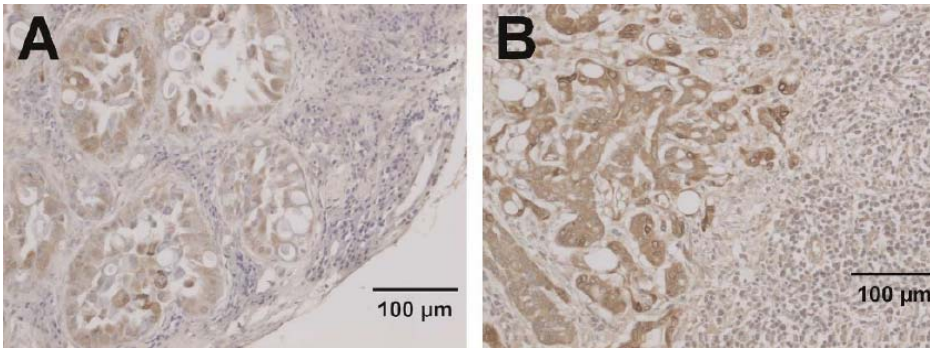

Figure 1. Prostate carcinoma: Immunoperoxidase-DAB for COX-2. Hematoxylin counterstain. A. Micropapillary subtype with a moderate degree of inflammation. B. Solid subtype with strong COX-2 staining.

The relationship between degree of inflammation and staining index is shown in Figure 2. The presence of inflammation (score 0 vs. score 1, 2 or 3) was significantly associated with a lower staining index for COX-2 (P=0.001) in both the micropapillary and solid histological subtypes. This was mainly due to a significantly lower number of positive cells (P=0.04) rather than to a lower intensity of staining (P=0.630). However, the degree of inflammation (scores 1 to 3) was not significantly associated with different staining indices. In general, when present, inflammatory infiltrates were homogeneously distributed throughout the entire tissue section on the slides.

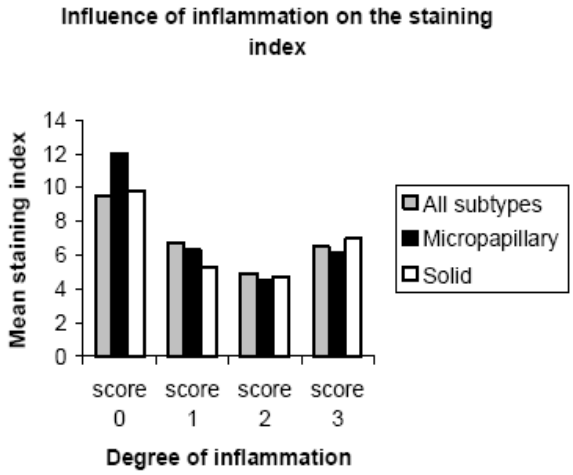

Figure 2. Relationship between degree of inflammation and staining index for all PCA samples and for the 2 most common histological subtypes (micropapillary and solid).

COX-2 expression in cultured cells

Results are shown in Figures 3 and 4. Baseline expression of COX-2 was barely detectable in the non-neoplastic CAPE cells, but was marked in the neoplastic ACE cells. This baseline COX-2 expression could be completely inhibited by blocking either PI3K, PKC or

ERK/MAPK in the ACE cells. In the CAPE cells, inhibition of ERK/MAPK by PD98059 induced COX-2 expression to clearly higher levels than baseline.
TNF-α and IL-6 had similar effects. Both cytokines induced higher COX-2 expression in the CAPE cells, compared to baseline. This induction was blocked by inhibition of the PI3K and PKC pathways. Blockage of the ERK/MAPK pathway did not decrease, but rather enhanced the inductive effect of TNF-α and IL-6 in CAPE cells. Conversely, in the ACE cells, TNF-α and IL-6 both caused a slight reduction in COX-2 expression. COX-2 expression was further reduced by blocking the PI3K pathway. Blocking the PKC and the ERK/MAPK left COX-2 expression unchanged compared to incubation with TNF-α or IL-6 alone.
Incubation of cell cultures with PMA increased COX-2 expression in both the non-neoplastic CAPE cell line and the neoplastic ACE cell line. In the CAPE cell line, this inductive effect of PMA could not be blocked by inhibition of any of the pathways. In the neoplastic ACE cells, blockage of the PKC pathway caused almost complete inhibition of COX-2 expression. Blocking other pathways did not alter induction of COX-2 expression by PMA.

EGF caused induction of COX-2 expression in CAPE cells similar to that caused by TNF-α and IL-6. This induction was only partially reduced by blocking the PI3K and PKC pathways. In the ACE cells, EGF caused no induction of COX-2 expression compared to control cells but partially reversed inhibition of COX-2 expression by inhibitors of the PI3K and ERK/MAPK pathways and completely reversed blockage by inhibitors of the PKC pathway.

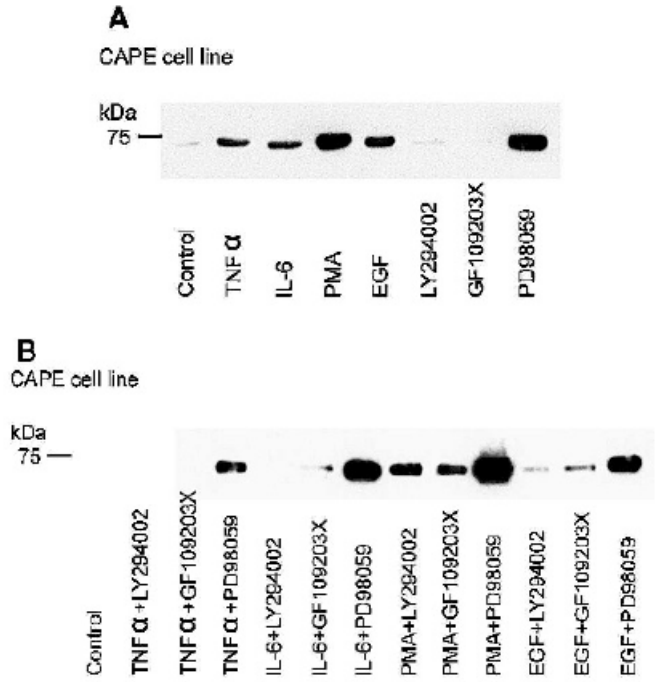

Figure 3. Western blot showing COX-2 expression in the non-neoplastic CAPE cell line after incubation with various combinations of stimulators and cell signaling pathway inhibitors.
Stimulators: TNFα = tumor necrosis factor alpha, IL-6 = Interleukin-6, PMA = tumor promoter phorbol 12-myristate 13-acetate, EGF = epithelial growth factor
Inhibitors: PD98059, inhibitor of Extracellular signal Related Kinase (ERK/MAPK), GF109203X, inhibitor of Protein Kinase C (PKC), and LY294002, inhibitor of Phosphatidyl Inositol-3 Kinase (PI3K).

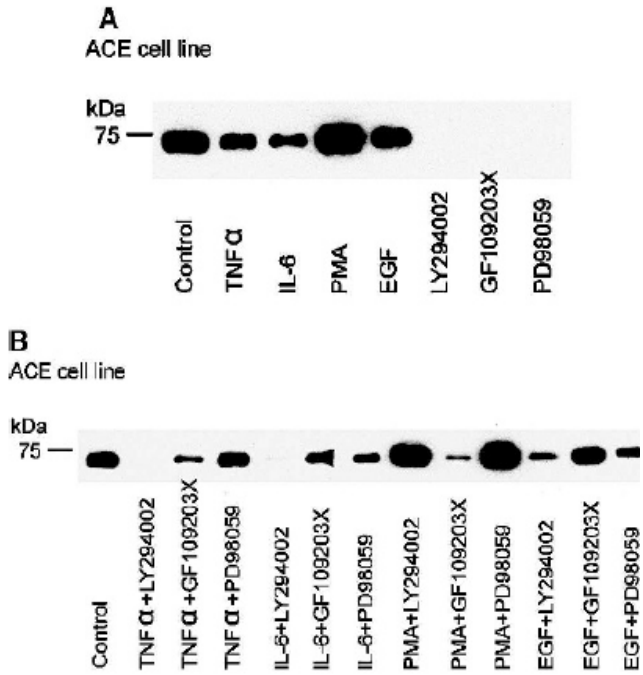

Figure 4. Western blot showing COX-2 expression in the neoplastic ACE cell line after incubation with various combinations of stimulators and cell signaling pathway inhibitors.
Stimulators: TNFα = tumor necrosis factor alpha, IL-6 = Interleukin-6, PMA = tumor promoter phorbol 12-myristate 13-acetate, EGF = epithelial growth factor
Inhibitors: PD98059, inhibitor of Extracellular signal Related Kinase (ERK/MAPK), GF109203X, inhibitor of Protein Kinase C (PKC), and LY294002, inhibitor of Phosphatidyl Inositol-3 Kinase (PI3K).

Discussion

The proportion of dogs with positive staining for COX-2 (89.3%) is consistent with findings in other studies in dogs [4,9] and in humans [5,6,11], in which 80-90% of prostatic tumors showed COX-2 expression. The classification of canine PCA into different histological subtypes and the finding in this study that many tumors consist of more than 1 morphological tumor subtype confirm the earlier described heterogeneous character of PCA in dogs [15,26]. Two subtypes (micropapillary and solid) clearly dominated, whereas the other subtypes occurred far less frequently.
The micropapillary subtype is characterized by tumor cells within the original glandular acini and an intact basement membrane, whereas the solid tumor type is characterized by tumor invasion and breach of the basement membrane and normal acinar structure, and therefore can be considered less well differentiated. There was a tendency in this study towards a higher proportion of less differentiated tumor subtypes (solid type) in castrated dogs, compared to

intact males. This finding is consistent with observations in humans, where androgen ablation often leads to development of less well-differentiated PCA (higher Gleason grades)[27]. Also consistent with findings in humans was the tendency towards lower COX-2 expression in PCA in castrated dogs compared to intact males. A similar infiltration of inflammatory cells also has been observed in humans as a consequence of androgen ablation therapy[28]. However, the COX-2 staining indices found in this study did not correspond to what would be expected based on the reported COX-2 expression in human PCA. The COX-2 expression found in the canine prostatic tumors examined in this study did not vary with the degree of differentiation of the tumors, whereas in humans intensity of COX-2 staining has been found to be higher in less differentiated tumors than in well-differentiated tumours[11]. Furthermore, canine tumors without inflammation had a significantly higher staining index than did tumors with inflammation, although no relationship between the degree of inflammation in these tumors and COX-2 staining could be detected. In humans, COX-2 expression correlates with the presence of local chronic inflammation[12], and it is even hypothesized that inflammation may initiate and promote prostate cancer[29]. Findings in this study suggest therefore that factors other than inflammation are responsible for the induction in COX-2 expression in canine prostate cancer.

In vitro, a clear difference was found between neoplastic and non-neoplastic prostate cells. Evidence of much higher COX-2 expression in neoplastic ACE cells than in non-neoplastic CAPE cells reflects the in vivo situation, where COX-2 expression is seen in neoplastic prostatic tissue but not in normal prostate tissue[4,9,30].

Cytokines TNF-α and IL-6 and the growth factor EGF all caused induction of COX-2 expression in the non-neoplastic CAPE cells, confirming earlier findings by others that COX-2 may be induced by a variety of cytokines and growth factors[31-33]. PD98059, an inhibitor of the ERK/MAPK pathway, also induced COX-2 expression in the CAPE cells. This suggests that the ERK/MAPK pathway may be important in suppressing COX-2 expression in prostate cells of non-neoplastic origin. Blockage of the PI3K and PKC pathways completely suppressed induction of COX-2 expression caused by cytokines TNF-α and IL-6, and greatly reduced induction caused by EGF, indicating that both pathways are necessary for induction of COX-2 by these cytokines and growth factors.

In the neoplastic ACE cells, TNFα and IL-6 slightly decreased COX-2 expression compared to baseline value. This finding correlates with the reduced COX-2 staining found in the presence of inflammation in the canine prostate carcinomas examined in this study, because TNF-α and IL-6 are important inflammatory cytokines. In addition, our findings show that endogenous COX-2 expression in the ACE cell line is dependent on all investigated pathways (PI3K, PKC and ERK/MAPK) functioning, beecause blockage of either 1 of these signal transduction molecules caused a significant inhibition of COX-2 expression to non-detectable levels.

In the presence of exogenous TNF-α and IL-6 however, COX-2 expression in the ACE cell line is maintained despite blockage of the PKC and ERK/MAPK pathways. The PKC pathway therefore seems to be involved in the regulation of COX-2 by TNF-α, IL-6 and EGF in normal prostate cells but not in neoplastic cells.

COX-2 expression stimulation of the ACE cells by EGF is partially blocked by inhibition of either PI3K or ERK/MAPK. Thus, while in the non-neoplastic cell line, COX-2 expression is increased by blocking ERK/MAPK, COX-2 expression in the neoplastic cell line is increased by stimulating this kinase.

The phorbol ester PMA was a strong inducer of COX-2 expression in this study. This confirms the findings of others[17]. PMA is known to activate the PKC signalling pathway[17]. Taken together, the results of the present study show that COX-2 is expressed in canine prostate carcinoma and is inversely correlated with the presence of inflammation in these tumors. This finding is confirmed in our in vitro study. Although COX-2 expression in non-neoplastic cells may be induced by cytokines and growth factors, inflammation (in vivo) and cytokines (in vitro) tend to decrease the already high baseline COX-2 expression found in neoplastic PCA cells. Although stimulation of PI3K and PKC lead to COX-2 expression in non-neoplastic prostate cells, only PI3K preserved this function in neoplastic cells. Moreover, although inhibition of ERK/MAPK in non-neoplastic cells induces COX-2 expression, inhibition of the same protein in neoplastic cells exerts a similar effect. These results point to changed signal transduction pathways in PCA leading to increased COX-2 expression. Additional studies are required to fully understand the mechanisms regulating COX-2 expression in canine prostate carcinoma.

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CHAPTER 4

Androgen receptor CAG repeat polymorphisms in canine prostate cancer

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Abstract

Background: Relatively shorter lengths of the polymorphic polyglutamine repeat-1 of the androgen receptor (AR) have been associated with an increased risk of prostate cancer (PC) in humans. In the dog, there are 2 polymorphic CAG repeat (CAGr) regions.
Objective: To investigate the relationship of CAGr length of the canine AR-gene and the development of PC.
Animals: Thirty-two dogs with PC and 172 control dogs were used.
Methods: DNA was extracted from blood. Both CAG repeats were amplified by polymerase chain reaction (PCR) and PCR products were sequenced.
Results: In dogs with PC, CAG-1 repeat length was shorter ($P = 0.001$) by an increased proportion of 10 repeats ($P = 0.011$) and no 12 repeats ($P = 0.0017$) than in the control dogs. No significant changes were found in CAG-3 length distribution. CAG-1 and CAG-3 polymorphisms proved not to be in linkage disequilibrium. Breed difference in allelic distribution was found in the control group. Of the prostate-disease sensitive breeds, a high percentage (64.5%) of the shortest haplotype 10/11 was found in the Doberman, whereas Beagles and German Pointers had higher haplotype 12/11 (47.1 and 50%). Bernese Mountain dogs and Bouvier dogs both shared a high percentage of 11 CAG-1 repeats and 13 CAG-3 repeats. Differences in (combined) allelic distributions among breeds were not significant.
Conclusions and Clinical Importance: In this preliminary study, short CAG-1 repeats in the AR-gene were associated with an increased risk of developing canine PC. Although breed-specific differences in allelic distribution of CAG-1 and CAG-3 repeats were found, these could not be related to PC risk.

Introduction

The prostate is an androgen dependent organ[1]. Androgens mediate their effect through activation of the androgen receptor (AR). Upon binding of the biologically most active testosterone metabolite dihydrotestosterone (DHT), the proliferation, survival, and expression of differential markers of prostate epithelial cells is stimulated[2].
In humans, the activation of the androgen-AR axis is believed to play an important role in the development and progression of prostate cancer. Epidemiologic studies demonstrated that high plasma androgen concentrations are associated with a high incidence of prostate cancer.[3,4] Continuous administration of testosterone pellets to Nb rats promotes the development of prostate adenocarcinoma.[5] Androgen deprivation initially blocks the tumor growth in humans. However, an androgen independent tumor re-occurs eventually in these patients and leads to a uniformly lethal drug-resistant stage.[6] In this stage there is still expression and activity of AR in the recurrent prostate cancer cells,[7-9] which demonstrates the importance of the AR in the oncogenesis and the progression of prostate cancer.
The human AR gene is located on the X chromosome and is composed of eight exons. The first exon encodes the *N*-terminal domain which possesses transcriptional activity. This *N*-terminal transactivation domain of the AR gene contains 3 polyglutamine repeats and one polyglycine repeat. One of the polyglutamine (CAG) repeats is polymorphic and consists of 11 - 31 repeats in the germline DNA of the normal population.[10] The length of this repeat has been reported to correlate with the risk of prostate cancer. Humans with shorter CAG repeat length are at higher risk of developing prostate cancer compared to those with longer variants.[11-13]
The dog is the only non-primate species that develops prostate cancer spontaneously. Canine prostate cancer (cPC) shares several similarities with human prostate cancer. Both tumors are commonly found in older individuals,[14] tumor growth beyond the prostate is common and the distribution of distant metastases (bone and lung) is similar to that seen in humans.[15,16]
The role of androgens and their receptor in canine prostate cancer is not yet clear. Most canine tumors do not respond to androgen depletion.[17] Furthermore, an increased risk of prostate cancer is found in castrated male dogs and in the Bouvier des Flandres breed.[18] These findings may point to no inductive, or even a protective role of androgens in canine prostate cancer development. The fact that prostate tumors in non-castrated dogs are generally of a more differentiated type compared to the tumors in castrated dogs may further underline this hypothesis.[19]
Similar to the human, rat and mouse AR, the canine AR sequence contains 3 polyglutamine repeats and 1 polyglycine repeat.[20] Two of these $(CAG)_n$ microsatellites in the canine AR are reported to be polymorphic.[21] The first polymorphic CAG repeat (CAG-1) was found at a similar position as the human polymorphic CAG-I repeat, while the second polymorphic CAG repeat (CAG-3) was located at a position corresponding to that of the CAG-3 region in the rat.[20]
The aim of this study is to investigate whether polymorphisms in the CAG repeat (CAGr) of the canine AR gene can be related to the development of prostate cancer in dogs.

Material and methods

Subjects

For analysis of the allelic distribution of CAG repeat, blood samples were obtained from 32 dogs with prostate cancer and 172 male control dogs that were admitted to the Utrecht University Clinic for Companion Animals during 2002-2005. The group of dogs with cPC came from 17 different breeds, including 3 Bouviers des Flandres. All were older dogs with a mean age of 10.7 yrs (range= 6.7-13.5). Control dogs were selected from those referred to the hospital with reasons unrelated to the prostate. The control group consisted of 17 Beagles, 45 Bernese Mountain dogs, 30 Bouvier des Flandres, 31 Dobermans, 36 German Pointers, 6 Scottish Terriers, and 7 dogs of other breeds. DNA extraction was performed by a salt extraction method as reported previously.[22]

Primer development

The complete coding sequence of the canine androgen receptor was obtained from Genbank (gi:6578766; www.ncbi.nlm.nih.gov). Two primer pairs were used to amplify the two polymorphic repeats within exon 1 of the canine AR gene. One of each primer pair was attached to a M13 tail, which was used to introduce a phosphoramidite (FAM) dye attached to a M13 primer in order to label the PCR product (Table 1). The PCR product size for the first CAG repeat is 238 base pairs, and for the second CAG repeat 270 base pairs, both calculated if the CAG repeat was 11 repeats long.

Table 1. Primers used in this study

Primer	Sequence	Annealing Temperature (ºC)
CAG-1 forward	GTTTTCCCAGTCACGACGA ACCTGTTCCAGAGTGTGC[a]	57
CAG-1 reverse	TCCTCATCCAGAGCCAGGTA	
CAG-3 forward	CCAGCACCACCGGACGAGAATGA	64
CAG-3 reverse	GTTTTCCCAGTCACGACCGGCGGC CTCCCTTGCTCTC[a]	

[a]The underlined sequence denotes the M13-tail

Polymerase chain reaction (PCR)

The polymorphic CAG repeats of canine androgen receptor were amplified by PCR, using 25 ng of genomic DNA in a final volume of 15 µl PCR mixture, containing 1× PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.03 µM primer with M13 tail, 0.3 µM FAM-labeled M13 primer, 0.3 µM complementary primer and 0.3 units AmpliTaq Gold. The cycle conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 10 cycles

of denaturation at 95°C for 30 sec, annealing at specific temperatures (Table 1) for 15 sec, and extension at 72°C for 30 sec. This was followed by a further 25 cycles using a denaturation temperature of 92°C. Finally, an extension step for 10 min at 72°C was performed.

Estimation of repeat length
Two microliter of a 10-fold diluted PCR-product was mixed with 10 µl of formamide and 0.2 µl of a carboxytetramethylrhodamine (TAMRA)-labeled 500 base pair size standard[q] in a 96 optical well plate. Products were denatured at 95ºC for 3 min and immediately placed on ice for 5 min. The length of the PCR product was determined on an ABI 3100 Genetic Analyzer[r] and evaluated using the GeneScan Analysis program.

Sequencing analysis
Sequence analysis for the two different PCR products was performed at least twice, with independently amplified PCR products. Tercycle reactions were performed in a 10 µl volume and contained 2 µl of 10-fold diluted PCR product, 3.2 pmol primer and 1µl Big Dye Terminator v 3.0 Ready Reaction Mix[b] in 1× sequence buffer (400 mM Tris, 10 mM $MgCl_2$, pH 9.0). The tercycle consisted of 25 cycles of denaturation at 96ºC for 30 sec, annealing at 53ºC for 15 sec and extension at 60ºC for 2 min. Tercycle products were then purified using multiscreen 96-well filtration plates filled with Sephadex-gel G-50 superfine[s] and sequenced in 20 µl distilled water using the ABI 3100 Genetic Analyzer. The length of the AR-CAG repeats in each individual dog was analyzed by counting the number of CAG trinucleotides using the DNAstar software package[t].

Statistics
Analysis of data was performed using the statistical software SPSS (version 12.0). CAG repeat length distributions between the cPC and the control group were compared by Fisher's exact test and Mann-Whitney test with 95% confidence interval. In order to compensate for multiple testing, statistical significance was accepted for P-values ≤ 0.01. The linkage disequilibrium measure r^3 was calculated by formulae described elsewhere[u,33].

Results

Allelic distribution in the population
From the length of the PCR products obtained by GeneScan analysis, the length of the CAG-1 and CAG-3 repeats was calculated. This calculated number of trinucleotide repeats was confirmed by sequence analysis, with two independent PCR products for both repeats. For each repeat PCR, the estimation of one sample was inadequate for the assessment of the CAG

[q] LIZ™, Applied Biosystems Part No. 4322682
[r] Perkin Elmer Applied Biosystems, Foster City, CA
[s] Amersham Bioscience, Roosendaal, The Netherlands
[t] DNAstar Inc, Madison, WI
[u] Excel, Microsoft Corporation, Redmond, WA

length in tumor DNA and therefore excluded. The number of CAG repeats in the CAG-1 varied between 10 and 12 in our study group, whereas CAG-3 contained 11 to 13 repeats. Next, the allelic distribution for each repeat in both the prostate cancer and the control group was calculated (Table 2). A significant difference (P = 0.001) in the CAG-1 repeat length was found between the cPC dogs and the normal dogs. The proportion of 10 repeats was significantly increased (P = 0.011) and no 12 repeats were present in dogs with PC. No significant changes were found in the distribution of the CAG-3 length between control and dogs with PC.

Table 2. Allelic distribution in dogs with PCA and in normal dogs

CAG-1					CAG-3				
Length	N	PCA (%)	n	Normal (%)	Length	n	PCA (%)	n	Normal (%)
10	17	54.8	51	29.7	11	19	61.3	130	75.6
11	14	45.2	84	48.8	12	4	12.9	13	7.6
12	0	0.0	37	21.5	13	8	25.8	29	16.9
Total	31[a]	100.0	172	100.0	Total	31[a]	100.0	172	100.0

[a]In both CAG-1 and CAG-3 one sample for repeat length measurement was inadequate

From the 6 different alleles, 9 potentially different haplotypes can be constructed, denoted as CAG-1/CAG-3 repeats by numbers (Table 3). Haplotype 12/13 was not found in any of the samples investigated. Calculation of the sum of repeat lengths resulted in a variation of 21-24 repeats. However, no significant difference of the total repeat length was found between the prostate cancer group and the normal group (P = 0.243).

Table 3. Haplotype distribution in dogs with PCA and in normal dogs

	PCA dogs		Normal dogs	
	Frequency	Percentage	Frequency	Percentage
Haplotype				
10/11	8	26.7	35	20.3
10/12	1	3.3	2	1.2
10/13	8	26.7	14	8.1
11/11	10	33.3	60	34.9
11/12	3	10	9	5.2
11/13	0	0	15	8.7
12/11	0	0	35	20.3
12/12	0	0	2	1.2
12/13	0	0	0	0
Total	30[a]	100	172	100

[a]In 2 dogs 1 CAG repeat length could not be established

Comparison between breeds

Among the control dogs the difference of allelic distribution between breeds was examined (Table 4). For the polymorphic CAG-1 region, the Doberman and Scottish terrier showed a relatively larger proportion of the shortest repeat length (allele 10), compared to other breeds, and missed the longest allele 12. In contrast, German Pointers and Beagles had the biggest proportion of allele 12 and relatively less allele 10. With regard to CAG-3 repeats, the majority of the dogs in each breed had mostly the shortest allele 11. The Beagle, Scottish terrier and the group of "other breeds" did not have the longest allele 13, whereas the Bernese mountain dog and German Pointer did not have allele 12. The Bernese Mountain dog had the largest proportion of longest allele 13. No significant differences in the (combined) allelic distributions between breeds could be detected (Table 5).

Table 4. Difference in allelic distribution between breeds in normal dogs

	CAG-1 (%)			CAG-3 (%)		
Breed Length	10	11	12	11	12	13
Beagle (n = 17)	5.9	47.1	47.1	82.4	17.6	0.0
Bernese Mountain dog (n = 45)	24.4	64.4	11.1	55.6	0.0	44.4
Bouvier des Flandres (n = 30)	30.0	56.7	13.3	60.0	16.7	23.3
Doberman (n = 31)	67.7	32.3	0.0	93.5	3.2	3.2
German Pointer (n = 36)	8.3	41.7	50.0	97.2	0.0	2.8
Scottish Terrier (n = 6)	66.7	33.3	0.0	83.3	16.7	0.0
Other breeds (n = 7)	28.6	42.9	28.6	57.1	42.9	0.0

Table 5. Differences in haplotype between breeds in normal dogs.

	Haplotype							
Breed	10/11	10/12	10/13	11/11	11/12	11/13	12/11	12/12
Beagle (n = 17)	1	0	0	5	3	0	8	0
Bernese Mountain dog (n = 45)	5	0	6	15	0	14	5	0
Bouvier des Flandres (n = 30)	2	0	7	13	4	0	3	1
Doberman (n = 31)	20	1	0	9	0	1	0	0
German Pointer (n = 36)	2	0	1	15	0	0	18	0
Scottish Terrier (n = 6)	4	0	0	1	1	0	0	0
Other breeds (n = 7)	1	1	0	2	1	0	1	1

Discussion

In order to determine whether polymorphisms in the CAG repeat length (CAGr) of the AR gene can be related to the development and progression of PC in the dog as in humans,[11-13] we analyzed the length of AR-CAGr in genomic DNA of 32 dogs with PC and 172 male control dogs. Our results demonstrated that the CAG-1 region of the AR gene in the canine population contained 10-12 CAGr, whereas the CAG-3 region contained 11-13 CAGr. The range of the CAG-3 repeats in this study were somewhat narrower than those reported by

Shibuya et al[21], in which the CAG-1 also contained 10-12 repeats but the CAG-3 contained 10-13 repeats. Differences in study populations may explain this variability in CAGr range. In the study of Shibuya et al,[21] a larger number of breeds was included and 27 of the 80 dogs were female.
The allelic distribution of CAG-1 appeared to be significantly different between the dogs witih PC and the normal dog population. Similar to humans, [11,24-26] shorter CAGr were found more often in the canine PC group, with an overall lack of the longest length CAG-1. In humans, shorter CAGr in exon-1 of the AR gene has been suggested to play a cauasal role in PC development[11], because it encodes an AR with higher transcriptional activity[27]. However, all canine AR CAG-1 repeats are short when compared with the human length of 11-31 repeats in this allele[10]. Increased transcriptional activity fir the short canine CAG-1 repeat remains to be investigaed.

Reports about the inverse association between CAGr length of AR gene and PC risk in human are contradictory. Some studies found statistical significance,[11,24-26] whereas others failed to prove the same.[27-30] Giovannucci[31] summarized these discordance and proposed a "two pool" model, one of relatively aggressive, early onset "androgen-driven" prostate cancers and the other occurring at older ages which may be less driven by androgens and more related to pathologic processes such as oxidative insults. A recent study from the same group found that patients with higher plasma total or free testosterone concentrations had lower-grade cancer while those with lower plasma total or free testosterone concentrations had higher-grade cancer.[27] This fact implies a more protective role of androgenic stimulation in PC cells or even prevention of PC and was in agreement with our previous studies in which we observed that non-castrated dogs have a lower risk of PC and that PC in castrated dogs showed more often poorly differentiated histomorphology.[18,19] The features of canine PC, low incidence and lack of prostate marker screening, is supportive of an inverse association between CAGr length and PC risk in humans and is concordant with Giovannucci's suggestion. It is possible that those AR encoded by genes with shorter CAG-1 have higher transactivation function, which accelerates the transit of nascent cancer from the latent phase to a symptomatic phase.
The fact that the difference in the dog may be < 2 repeats is in concordance with a recent meta-analysis of 23 epidemiologic studies on the association between AR CAG and GGN polymorphisms and PC in humans[34]. In this study, the presence of shorter repeats was found to be modestly associated with PC risk, with the absolute difference in number repeats between cases and controls < 1 repeat. By far the main contribution in this meta-analysis is from Caucasian men. In a recent study by Lange et al[35], the authors failed to find such an association in African-American men. However, African-American men already have shorter CAGr compared to Western white and Asian men and have a higher incidence of PC.[32] In the European canine population, the Bouvier has been reported to have a higher risk of prostate cancer.[18] However, this effect of a shorter CAGr was not reflected in the allelic distribution of the two repeats that were investigated. Nevertheless, a breed difference in allelic disctribution was found. Doberman and German Pointer dogs have quite different allelic distribution despite the fact that both breeds are predisposed to benign prostate hyperplasia

(BPH) and PC.[18,36] Bernese Mountain dogs and Bouvier dogs shared a high percentage of 11 CAG-1 repeats and 13 CAG-3 repeats, whereas of the two breeds, only the Bouvier is reported to have a higher PC risk. The relatively low number of other breeds represented in this study cannot exclude other breed differences.
In summary, our study showed an overall lack of 12 repeats and an increased short CAG-1 repeat within exon 1 of the androgen receptor gene in the canine PC group, which was significantly different from the control group. No significant differences were found in the third CAG polymorphic region or in the combined CAGr length between cases and control. CAG-1 and CAG-3 polymorphisms were found not to be in linkage disequilibrium. Although breed-specific differences in allelic distribution of CAG-1 and CAG-3 repeates were found, these could not be related to PC risk. More studies are needed to further elucidate the role of this in the development of PC.

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CHAPTER 5

Highest plasma testosterone and DHT concentrations in Bouviers des Flandres with short CAG-I and long CAG-III repeats in the androgen receptor gene

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Abstract

Epidemiological studies of prostatic disease in dogs have found the Bouvier des Flandres breed to be predisposed to developing prostate carcinoma (PCA). In humans, ethnic groups with a higher prevalence of PCA have been found to have higher serum concentrations of androgens and shorter polyglutamine (CAG) repeat lengths in the androgen receptor (AR) gene. Dogs also have two polymorphic CAG areas in the AR gene (CAG-I and CAG-III). Shorter CAG-I lengths have been associated with an increased risk of developing PCA. The objective of this study was to determine whether differences in androgen concentrations are related to the increased risk of developing PCA in the Bouvier des Flandres compared to dogs of other breeds and whether there is a correlation between CAG repeat length and plasma androgen concentrations. Plasma samples from 46 healthy Bouviers and 53 healthy dogs of other breeds was analysed for concentrations of testosterone (T), dihydrotestosterone (DHT) and androstenedione (A). In addition in the Bouvier group the length of both polymorphic polyglutamine repeats were measured. No significant differences in the plasma concentration of androgens between the Bouvier group and the control group were found. In the Bouvier group shorter CAG-I and longer CAG-III repeat lengths were associated with lower concentrations of androgens. As the shorter CAG-I repeat is associated with an increased PCA risk, higher androgen concentrations may protect against the development and progression of PCA in line with findings that castration has a permissive role for the development of PCA in Bouvier dogs.

Introduction

An epidemiologic study of prostate disease in dogs has shown that the Bouvier des Flandres has a more than 8 times higher risk of developing prostate carcinoma (PCA) compared to a reference population of dogs (Teske et al., 2002). In other reports, middle to large breed dogs are reported to be overrepresented among dogs with PCA (Bryan et al., 2007; Cooley and Waters, 2001).
In humans there is a well documented difference in incidence of PCA between different ethnic groups (Bostwick et al., 2004; Winters et al., 2001). African-American men have the highest incidence of prostate cancer in the world (Bostwick et al., 2004; Jemal et al., 2005). However, it is uncertain whether this difference is truly due to ethnicity, as a number of other factors may also explain the predisposition of a certain group to PCA. These include differences in exposure to carcinogenic substances (particularly caused by dietary differences), differences in detection and genetic differences (Bostwick et al., 2004). Only the latter can be of importance in dogs, since there are no breed-related differences in nutrition and veterinary treatment in the dog population. In humans, differences in serum concentrations of androgens are a possible cause of the racial differences in prostate cancer risk. In African-American men higher concentrations of testosterone and sex hormone-binding globulin (SHBG) have been found, compared to the Caucasian population (Abdelrahaman et al., 2005; Winters et al., 2001). It has been hypothesized that SHBG can activate androgen responsive genes (Winters et al., 2001). Other race-related differences reported include the activity of the enzyme 5α-reductase (Ross et al., 1992; Wu et al., 2001), concentrations of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (Platz et al., 1999; Winter et al., 2001) as well as differences in the microsatellite alleles at the androgen receptor (AR) locus. In particular the length of CAG repeats in these microsatellite alleles may be a risk factor for the development of PCA (Coetzee and Ross, 1994; Giovannucci et al., 1997). African-American men were found to have shorter CAG repeats (Platz et al., 2000) and Japanese men longer CAG repeats (Ekman et al., 1999) compared to white men.
The objectives of this study were to investigate whether the higher incidence of PCA in the Bouvier des Flandres compared to dogs of other breeds may be associated with a higher plasma concentration of androgens in this breed. In addition, the relationship between the length of the CAG repeats in the AR gene and the plasma concentration of androgens was investigated.

Materials and methods

Animals
Blood samples were collected from 99 intact male dogs. Approximately 4 ml of blood was obtained from the cephalic or jugular vein of each dog and transferred to EDTA-coated tubes. All dogs were either healthy dogs (Bouviers des Flandres, recruited through breed club internet websites and discussion forums or by direct contact with breeders) or dogs presented to the clinic (Department of Clinical Sciences of Companion Animals, Faculty of Veterinary

Medicine, Utrecht University) for other conditions than prostatic disease. One group consisted of 46 Bouviers des Flandres while the other group consisted of 53 other dogs of different breeds. After centrifugation the plasma samples were stored at -20°C until hormone measurement.

Hormone assays

Testosterone
Total testosterone was measured using a Coat-A-Count solid phase radioimmunoassay kit obtained from the Diagnostic Products Corporation (Los Angeles, CA). Before the assay, 0.25 ml of plasma was extracted with 1 ml ether. After centrifugation the aqueous phase was frozen and the ether extract was decanted, evaporated to dryness and dissolved in 0.25 ml of buffer PBS with 0.5% (w/v) BSA.
The intra-assay coefficient of variation was 16.6% and the sensitivity was 0.14 nmol/l. The accuracy of the assay, measured by recovery, was 91.2%. Cross-reactivities were as follows: 4-estren-17-ol-3-one (20%), 11-ketotestosterone (16%), 5α-dihydrotestosterone (3.3%), 19-hydroxyandrostenedione (2.0%), methyltestosterone (1.7%) and 4-estren-7x-methyl-17B-ol-3-one (1.1%).

Androstenedione
Androstenedione was measured directly using a Coat-A-Count radioimmunoassay obtained from the Diagnostic Products Corporation (Los Angeles, CA). The intra-assay coefficient of variation was 4.2% and the sensitivity was 0.14 nmol/l. The cross-reactivities were as follows: testosterone (1.49%), 5α-dihydrotestosterone (0.212%), DHEA (0.164%), progesterone (0.160%) and adrenosterone (0.135%).

Dihydrotestosterone
DHT was measured after extraction using a radioimmunoassay kit obtained from Intertech (Strassen, Luxembourg). From each plasma sample, 300 µl was extracted by 3 ml of diethylether. The tubes were vortexed for 2 min and then centrifuged at 2000 rpm for 5 min. The aqueous phase was frozen and the ether extract was decanted and evaporated to dryness. After adding PBS buffer, vortexing and adding 50 µl oxidation solution, the tubes were incubated at 37°C for 20 min. The solution was extracted again by 3 ml mixture of hexane/ethanol 98/2. The tubes were vortexed, centrifuged and 2 ml of the organic layer was transferred into glass tubes and evaporated to dryness.
The sensitivity of the assay was 0.07 nmol/l, the intra-assay coefficient of variation was 6%. The cross-reactivities were as follows: estradiol (1.20%), estriol (0.04%), DES (<0.01%), pregnenolone (<0.01%), progesterone (<0.01%), testosterone (<0.01%) and aldosterone (<0.01%).

Androgen receptor gene polyglutamine repeats
The length of the polyglutamine repeats of each polymorphic region (CAG-I and CAG-III) were measured as described elsewhere (Lai et al., 2008b). Briefly, DNA was extracted from

blood, both CAG repeats were amplified by polymerase chain reaction (PCR). The length of the PCR products was determined on an ABI 3100 Genetic Analyzer.

Statistical analysis

Analysis of data was performed by the statistical software SPSS 15.0 for Windows (Statistical Package for Social Sciences, SPSS Inc, Chicago, IL, USA). Mean hormone concentrations in the Bouvier group and the control group were compared for significant differences using an independent sample t-test, after testing for normality with the one-sample Kolmogorov-Smirnov test. The correlation between age and androgen concentrations and between the different androgens was analysed by linear regression analysis. The relationship between CAG repeat length and serum androgen concentration was analysed by bivariate correlation analysis (Spearman's rho, 2-tailed). A P-value below 0.05 was considered significant.

Results

Animals

The median age was 5.9 years in the Bouvier des Flandres group (range 8 months to 11 years) and 3.2 years in the control group (range 11 months to 12 years). The control group consisted of 26 different breeds. The most represented breeds were the Labrador retriever (6 dogs), the German Shepherd dog (5 dogs) and cross breed dogs (5 dogs).

Hormones

In the Bouvier group, the mean plasma testosterone concentration was 14.53 ± 8.90 nmol/l (mean ± SD). In the control group the testosterone concentration was 13.49 ± 9.98 nmol/l (Fig. 1). There was no statistically significant difference between the groups (P = 0.623). The mean plasma DHT concentration was 1.27 ± 0.74 in the Bouvier group and 1.08 ± 0.87 in the control group. The difference between groups was not significant (P = 0.234). The mean plasma androstenedione concentration was 5.30 ± 4.57 nmol/l and 5.07 ± 6.19 nmol/l in the Bouvier des Flandres and control groups, respectively. There was no statistically significant difference between the groups for the androstenedione concentration either (P = 0.867).

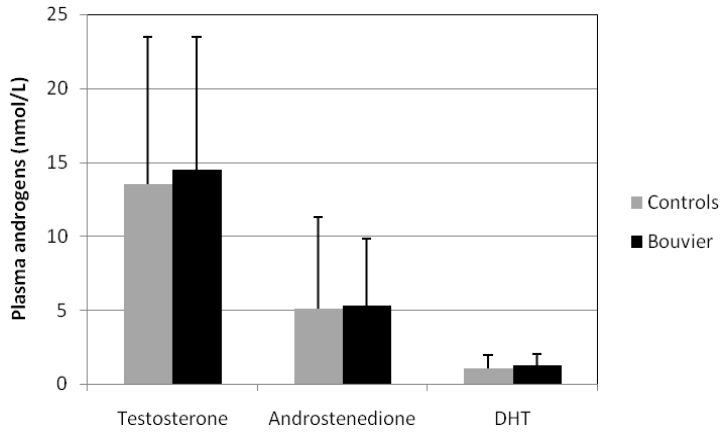

Figure 1. Plasma androgen concentrations in the Bouvier des Flandres and control groups (Mean ± SD).

No significant correlation between age and the concentrations of testosterone and DHT was found in the controls or the Bouviers. However, a significant positive correlation was found between age and androstenedione concentrations in the control group ($P = 0.036$), which was not found in the Bouvier group ($P = 0.172$). Furthermore, a significant positive correlation between testosterone and androstenedione concentrations was found in the Bouvier group ($P < 0.01$), which was not found in the control group ($P = 0.85$). Similarly, a significantly positive correlation was found between androstenedione and DHT in the Bouvier group ($P < 0.01$) but not in the control group ($p = 0.31$).

Androgen receptor gene polyglutamine repeats

In the first polymorphic region (CAG-I), 10 Bouviers had 10 repeats, 28 had 11 repeats and 3 had 12 repeats. The mean (± SD) concentration of testosterone, DHT and androstenedione for each of these groups is shown in Table 1.

Table 1. Plasma androgen concentration related to the length of CAG-I (mean ± SD)

Length of CAG-I repeat	*Testosterone (nmol/L)*	*DHT (nmol/L)*	*Androstenedione (nmol/L)*
10	11.29 ± 7.09	0.99 ± 0.33	5.17 ± 5.05
11	13.69 ± 10.05	1.19 ± 0.59	4.12 ± 3.00
12	21.12 ± 5.14	2.63 ± 1.56	12.58 ± 10.65

In the other polymorphic region (CAG-III), 30 Bouviers had 11 repeats, 5 had 12 repeats and 6 had 13 repeats. The mean (± SD) concentration of testosterone, DHT and androstenedione for each of these groups is shown in Table 2.

Table 2. Plasma androgen concentration related to the length of CAG-III (mean ± SD)

Length of CAG-III repeat	*Testosterone (nmol/L)*	*DHT (nmol/L)*	*Androstenedione (nmol/L)*
11	15.62 ± 9.61	1.42 ± 0.77	5.22 ± 4.63
12	9.85 ± 7.84	0.87 ± 0.33	2.11 ± 1.83
13	6.96 ± 3.80	0.88 ± 0.34	6.25 ± 6.43

Individual data and means for testosterone and DHT are plotted as a function of CAG repeat lengths (Fig 2 and 3). There was a significant positive correlation between the length of CAG-I and the plasma concentration of DHT, whereas the length of CAG-III was negatively correlated to the plasma concentration of testosterone and DHT. No significant correlation was found between CAG-I or CAG-III length and the concentration of androstenedione.

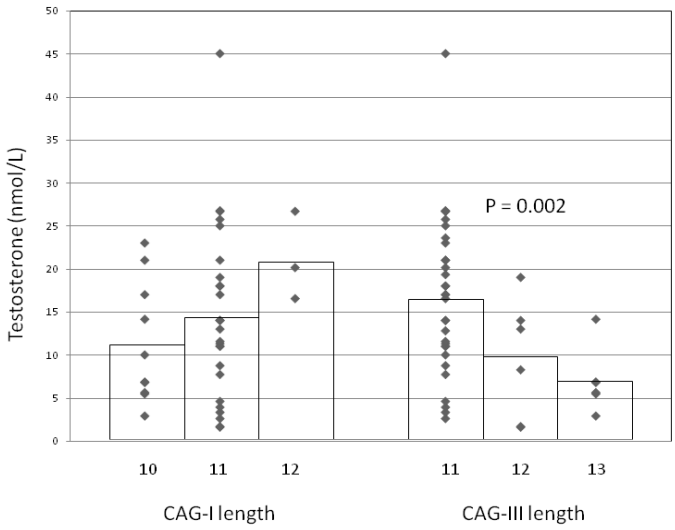

Figure. 2. Correlation between CAG repeat length and plasma testosterone concentration. The lozenges (◆) represent individual results. The boxes show the mean for each group. Where a significant ($P < 0.05$) correlation was found using Spearman's rho correlation analysis, the P value is indicated.

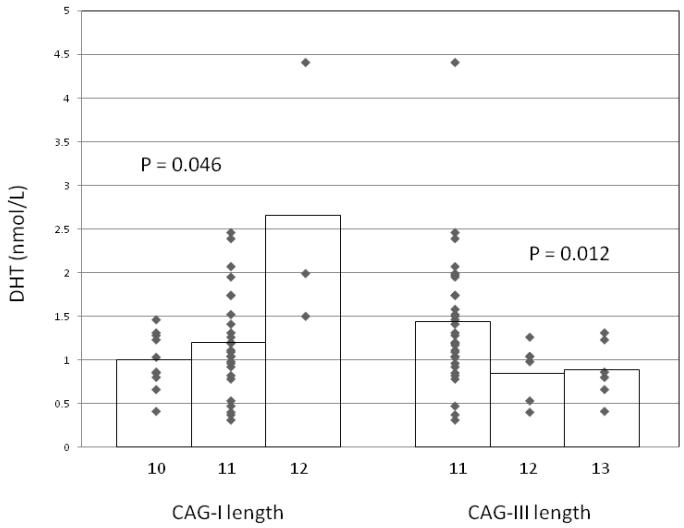

Figure. 3. Correlation between CAG repeat length and plasma DHT concentration. The lozenges (◆) represent individual results. The boxes show the mean for each group. Where a significant ($P < 0.05$) correlation was found using Spearman's rho correlation analysis, the P value is indicated.

Discussion

As most of the studies on androgen concentrations in the dog present their data in non-SI units for comparative reason our plasma testosterone concentrations found in this study equal 4.03 ± 2.59 ng/ml (Bouvier) and 3.14 ± 3.23 ng/ml (control). These results are higher than the mean testosterone concentrations in normal dogs reported in other studies where results ranged from 2.1 to 3.1 ng/ml (Corrada et al., 2004; Kamolpatana et al., 1998; Kawakami et al., 2001; Shimomura et al., 2004; Tsutsui et al., 2001), but lower than in two other studies with mean testosterone concentrations of 4.5 ng/ml (Hydbring-Sandberg et al., 2004) and 4.6 ng/ml (Knol et al., 1992). The present study included many more dogs than the previous reports mentioned, which all had less than 10 dogs. The variation in the testosterone concentration found in this study is larger in comparison to previous reports (Corrada et al., 2004; Kamolpatana et al., 1998; Kawakami et al., 2001; Shimomura et al., 2004; Tsutsui et al., 2001) although the two studies with higher reference values for testosterone also had higher standard deviations (Hydbring-Sandberg et al., 2004). Our study using a larger group of dogs emphasizes the larger variation of plasma testosterone concentrations in the dog. Whether this is due to variation in sex hormone binding globulin (SHBG) concentrations in the dog is unknown.

In humans, it has been hypothesised that the serum SHBG concentrations might be an important factor initiating the development of prostate cancer (Winters et al., 2001) and

further studies to examine the serum concentration of SHBG in Bouviers may yield additional information on possible causes for the apparent predisposition of this breed for prostate cancer. Although canine SHBG can be measured (Rhodes et al., 2000), human SHBG radioimmunoassays cannot be used as the molecular structure of canine SHBG differs too much from human SHBG.

In a recent study, CAG repeats in the AR gene were examined in dogs with and without PCA (Lai et al., 2008b). Similarly to the results found in humans, it was found that longer CAG-I repeats may have a protective effect and shorter repeats may assist in the development of PCA. No difference was seen in the length of CAG-III between dogs with PCA and those without. Despite a high percentage of short CAG-I repeats and long CAG-III repeats in the Bouvier des Flandres, no significant differences was found between this breed and other breeds of dogs. Therefore CAG length does not explain the apparent predisposition of Bouviers des Flandres for PCA.

The correlation found in this study between shorter CAG-I repeats and of longer CAG-III repeats with lower plasma testosterone and DHT concentrations may reflect differences in the effect of these repeats on recruiting of coactivators that play a role in transactivation of DHT-responsive genes. Moreover they may indicate a possible protective effect of androgens on PCA development, which is in agreement with previous findings that castrated dogs have a higher risk of developing PCA and that PCA in castrated dogs is less well differentiated than in intact males (Lai et al., 2008a; Teske et al., 2002). The mechanism of this protective effect is not known. In humans, androgens are required for the development and progression of PCA and treatment to eliminate androgens is effective in inhibiting progression and preventing the development of PCA (Huggins and Hodges, 2002; Thompson et al., 2003). However, there is also evidence that lower concentrations of androgens are related to higher histological grades of PCA (Platz et al., 2005; Thompson et al., 2003).

In conclusion, differences in plasma concentrations of androgens cannot explain breed differences in the risk of developing PCA in dogs in the same way as they are related to ethnic differences in men. Shorter CAG-I repeats in the Bouvier des Flandres, a breed predisposed to the development of PCA, are associated with a higher risk of PCA and lower concentrations of androgens, suggesting a possible protective role of androgens in the development of PCA.

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CHAPTER 6

Nd:YAG surgical laser effects in canine prostate tissue: Temperature and damage distribution.

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Abstract

An *in vitro* model was used to predict short-term, laser-induced, thermal damage in canine prostate tissue. Canine prostate tissue samples were equipped with thermocouple probes to measure tissue temperature at 3, 6, 9 and 12 mm depth. The tissue surface was irradiated with Nd:YAG laser in contact or non-contact mode for up to 20 s, using powers from 5 to 20 W. Prediction of thermal damage using Arrhenius theory was discussed and compared to the *in vitro* damage threshold, determined by histological evaluation. Threshold temperature for acute thermal tissue damage was 69 ± 6 ^{o}C (means ± sd), irrespective of exposure time. Contact mode laser application caused vaporization of tissue, leaving a crater underneath the fiber-tip. Mean extent of tissue damage underneath the vaporization crater floor was 0.9 ± 0.6 mm after 5, 10 or 20 s of contact mode laser irradiation at 10 W, whereas 20 W non-contact exposure up to 20 s causes up to 4.7 ± 0.2 mm coagulation necrosis. It was concluded that short-term acute thermal tissue damage can be comprehensively described by a single threshold temperature.

Introduction

Prostate surgery has several indications in dogs, such as the treatment of prostatic cysts or abscesses, and studies are being performed concerning the surgical treatment of prostate carcinoma (L'Eplattenier *et al* 2006). Radical (total) prostatectomy is often not an option in dogs because of the high incidence of urine incontinence after surgery, probably because of damage of the nerves and vessels located in the capsule of the canine prostate (Gordon 1960, Basinger *et al* 1987, Hardie *et al* 1990). A subcapsular debulking prostatectomy has been performed in dogs, preserving urinary continence by sparing the prostatic urethra and the prostate capsule (Robertson and Bojrab 1984, Rawlings *et al* 1994).
The extent of tissue damage in laser procedures is mainly dependent on the power density (W/cm^2) of the laser beam at the tissue surface and the irradiation time. Differences in tissue interaction due to changes in power density may be utilized in contact versus non-contact mode usage of a fiber-guided Nd:YAG laser (Shapshay 1987, Janda *et al* 2003). In contact mode, the high energy density at the fiber tip causes rapid carbonization of tissue. Carbonized tissue strongly absorbs laser irradiation leading to rapid heating and subsequent vaporization of tissue which enables tissue resection. Further heat distribution in the underlying tissue is mainly dependent on conduction and diffusion of the heat produced at the carbonized site. During laser resection of tissue the produced heat causes coagulation at the cut-surface so bleeding is reduced to a minimum. The non-contact mode, or free beam, technique applies lower energy density to avoid such superficial alterations of tissue, and permits deeper light-penetration and subsequent larger coagulated volumes (Motamedi *et al* 1995, Orihuela *et al* 1995, Lippert *et al* 2003). Apart from tumor coagulation and interstitial thermotherapy, the free beam is appreciated for its ability to conduct hemostasis (Ventrucci *et al* 2001, Mahoney and Shapshay 2005).
There is a need for knowledge about the extent of thermal damage caused by laser resection of prostate tissue and free beam hemostasis, for the laser to be used in prostate surgery in a safe and effective way. Most studies on Nd:YAG laser surgery on the dog prostate are based on transurethral laser applications for the treatment of benign prostatic hyperplasia (BPH) in men, in which the prostatic urethra is often destroyed and prolonged catheterization is required for re-epithelization. Transurethral 40-60W Nd:YAG contact mode vaporization of prostate tissue, using a moving side-firing contact probe, caused a relatively small coagulation zone of up to 2 mm underneath the carbonized surface (Muschter and Perlmutter 1994). Contact mode laser incision effects (bare fiber or contact probe) have been evaluated for several tissues *in vivo* (Shapshay 1987, Mecke *et al* 1991, Laranne *et al* 1997) and *in vitro* (Weber *et al* 1991, Perry *et al* 1997, Janda *et al* 2003), but not for prostate tissue. Results of these studies show minimal thermal tissue effects in depth, probably due to power-loss caused by carbonization and vaporization at the surface (Verdaasdonk *et al* 1990).
Aims of the present study were to set up an *in vitro* model for laser induced thermal tissue damage and to determine safe surgical margins prior to a clinical study investigating subcapsular laser prostatectomy (L'Eplattenier *et al* 2006). The effects of bare-end fiber contact and non-contact laser application on canine prostate tissue were investigated during

exposure times of up to 20 s, in contrast to existing literature describing other techniques (i.e. transurethral side-firing probes) using longer irradiation times (minutes to hours).

Materials and methods

Temperature distribution was measured during contact and non-contact mode laser application on *ex vivo* canine prostate tissue samples. The expected onset of thermal damage for *in vivo* tissue was estimated using the combination of measured temperature distribution in time and Arrhenius parameters, commonly used for damage prediction in prostate tissue. In addition, a threshold temperature for acute thermal damage in canine prostate tissue was determined through histological examination of *ex vivo* laser-induced thermal damage.

Tissue samples

Prostate tissue (< 12 hours post mortem) was collected from dogs without prostatic disease and was either stored at 5°C to be used for the experiment on the same day or stored at -20°C to be used on a later time. Sample sizes were approximately 3 - 5 cm^3. Only fresh samples (<8 hours post mortem, not previously frozen) were used for histological evaluation.

Temperature distribution in prostate tissue samples

A 1064 nm Nd:YAG laser (Medilas 40 N, MBB-Medizintechnik GmbH, München, Germany) was used to irradiate prostate tissue samples. Laser energy was applied through an optical fiber (600 µm diameter; Ultraline, Heraeus LaserSonics, Milpitas, CA). Four K-type thermocouple probes (0.26 mm diameter, response time < 20 ms) were inserted in the tissue samples through 18 G hypodermic needles fixed in a spacing device. The thermocouple probes were placed at 3, 6, 9 and 12 mm below the tissue surface (Figure 1). For non-contact mode, the laser fiber was positioned 6 mm above the tissue-surface area under which the thermocouple array was placed. This distance was chosen so that the highest power used (20 W) did not cause charring of the tissue surface. If charring nevertheless did occur, the measurements were discarded. For contact mode, the fiber tip was positioned against the tissue surface.

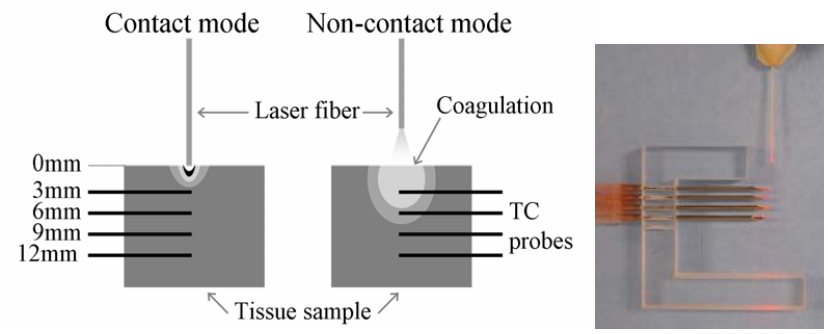


Fig 1: Experimental setup and spacing device for temperature measurements during contact and non-contact mode laser irradiation of *ex vivo* prostate tissue. Also displayed are indications of theoretical difference in coagulation and thermal damage distribution. TC: thermocouple.

Thermocouple probes were connected to a thermocouple-amplifier and temperature recordings were made through an AD-converter (Labjack U12, Pimzos Pinckard, The Netherlands) on a computer, resulting in temperature-time curves for each thermocouple probe. Probes and amplifier were calibrated using a precision Hg-thermometer (0.5^{o}C/div) and hot water bath.
Apart from the surrounding tissue, the thermocouple probes are also heated directly by laser irradiation. This phenomenon is visible as a sudden rise or fall in measured temperature value when the laser is turned on or off, respectively (Figure 2). This measurement artifact was corrected for, using the fact that the response time for laser-induced temperature change is much faster for the small thermocouple probes compared to tissue (Verdaasdonk *et al* 1991). All temperature recordings were corrected for this thermocouple artifact by separating the thermocouple response from the tissue response via a non-linear curve-fit of the tissue-cooling curve after the laser is turned off. The *measured* tissue cooling curve is assumed to be an exponential function: $T_m(t)=A+Be^{-Ct}+De^{-Et}$ (A-E: constant parameters), in which Be^{-Ct} represents the gradual tissue cooling component and De^{-Et} represents the rapid thermocouple cooling component (following laser switch-off) of the curve. The *calculated* (corrected) tissue temperature after laser switch-off is: $T_c(t)=A+Be^{-Ct}$ (Figure 2).

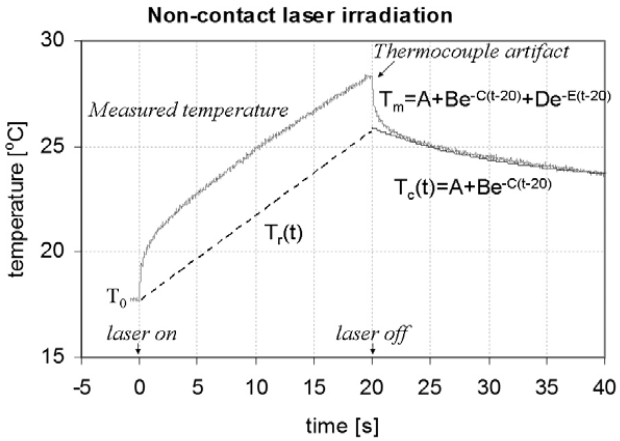


Figure 2: Example of a temperature recording at 6 mm tissue depth during 20 s non-contact mode laser irradiation, showing the measured temperature in time, the thermocouple artifact, the corrected cooling curve (t>20 s) and the calculated tissue temperature during laser exposure (dashed line). T_m: measured temperature. T_c: calculated temperature (t>20 s). T_r: reconstructed temperature during laser exposure.

It is assumed that at constant laser power, tissue temperature rises linear with time during short (up to 30 s) irradiation intervals, because the thermal relaxation time of tissue is much longer than the irradiation time (Manns *et al* 1998). Therefore, tissue heating during the irradiation period ($T_r(t)$) can be easily reconstructed using the corrected temperature at laser switch-off after a known irradiation interval (Figure 2). Mean temperature of the tissue samples at start was: $T_0=27 \pm 2$ ^{o}C. In non-contact mode experiments, temperature increase was recorded for 20 s of laser irradiation at 5, 10, 15 and 20 W laser settings. In the contact mode experiment, temperature increase was recorded for 5, 10 and 20 s of 10 W contact

mode laser application. The measured temperature increase was adjusted to body temperature (T_0+T_{adjust}=38^{o}C at t=0 s) to simulate *in vivo* situations.
The measured divergence half-angle of the laser beam, its transverse intensity typically being distributed in a Gaussian shape, was 9.4 ± 0.9^o (NA=0.16 ± 0.02), measured using thermal print paper at 3 cm distance from the fiber tip. The abovementioned laser powers were power settings of the laser device (Medilas 40N). The output-power of the laser fiber was measured using a standard power meter. The measured transmission of the laser fiber was 87 ± 0.5 %. Thus, the actual power output of the laser fiber with the applied settings of 5, 10, 15 and 20 W was 4.3, 8.8, 13.0 and 17.5 W respectively. Corresponding fluence rates are 139, 284, 419 and 565 W/cm^2 in the non-contact setup and up to 3.1 kW/cm^2 in the contact mode setup. When applicable, the experimental data are displayed as the actual power-output of the fiber.

Prediction of thermal damage
The most extensively used empirical model to describe time-dependent, temperature-induced tissue damage is derived from the temperature dependence of molecular reaction rates described by the Arrhenius parameters. This temperature relation for reaction speed was used by Henriques (1947) to formulate the Arrhenius damage integral, in which tissue damage is modeled as a first order rate process. The integral describes the relation between accumulated tissue damage and both temperature and time:

$$\Omega = A_f \int_0^{\tau} e^{-E_a / RT(t)} dt \qquad (1)$$

Ω represents the logarithm of the ratio of the original concentration of native state tissue constituent to the remaining concentration after a time τ. For instance, Ω=1 means that 63% of the tissue molecules are damaged, which is assumed to produce coagulation necrosis. A_f is the frequency factor; a measure of the molecular collision-rate (s^{-1}). E_a is the activation energy barrier (J mol^{-1}) for molecules to denature. R is the universal gas constant (8.31 J mol^{-1} K^{-1}) and T(t) the tissue temperature in time.
Arrhenius parameters for skin injury are frequently used in prostate tissue heating models (Prapavat *et al* 1996, Bolmsjo *et al* 1998, Huidobro *et al* 2004). A_f=3.1*10^{98}s^{-1} and ΔE_a=6.28*10^5 J mol^{-1}, describing necrosis threshold temperature for pig skin (Henriques 1947), are most widely used.

Histological evaluation of ex vivo laser induced thermal damage
Acute temperature induced irreversible tissue damage (i.e. thermal coagulation) is macroscopically visible as whitening of the tissue. Microscopic investigation shows that the border of this bleached zone coincides with the border of coagulation damage (Pearce and Thomsen 1995). With short exposure times, and thus fast heating rates, this border represents the transition zone of necrosis to viable tissue (Janda *et al* 2002). To investigate laser-induced tissue damage in our experiments, fresh prostate tissue samples (< 8 hours post mortem) were heated to 38^{o}C in Ringer's lactate and irradiated at several power and time settings. These fresh tissue samples were sectioned at the irradiated spot and the extent of coagulation (bleached zone) was measured macroscopically, using a caliper. Samples were also stored in

4% buffered formaldehyde to be embedded in paraffin, sliced and stained with hematoxylin and eosin (HE) for microscopic evaluation.

Statistics
Statistically derived experimental results are given as means ± sd. Curve-fits of the experimental results were made using the mean values. Differences in results between depths were tested for significance using Students t-test and were considered significant when $p<0.05$ (two-tailed).

Results

Tissue samples
A paired samples t-test (fresh versus frozen-thawed, $p=0.35$) was used on the non-contact results (see Figure 3) of all depths per laser power (16 groups with equal distributions of fresh versus frozen-thawed samples). No significant differences were found between results obtained from frozen-thawed tissue and results obtained from fresh tissue at the various depths and power settings. Therefore, all samples in this study are considered to have comparable optical and thermal properties and were treated as one group.

Temperature distribution in prostate tissue samples
Temperature rise after *20 s non-contact mode* laser exposure varied linear with applied laser power at every depth ($R^2 \geq 0.97$; Figure 3). Temperatures differed significantly between depths (t-test; $p<0.0005$). Temperature below the tissue surface decreased with tissue depth in an exponential fashion. The reconstructed temperature increase in time at 3, 6, 9 and 12 mm depth is displayed in Figure 5.
During *10 W contact mode* laser exposure, tissue temperature increased linearly with time at 6, 9 and 12 mm tissue depth ($R^2>0.99$; Figure 4). This linearity was less evident at 3 mm tissue depth, where $R^2=0.93$. Measured temperatures significantly differed between depths ($p<0.005$). Tissue temperature decreased with distance from the surface in an exponential fashion.

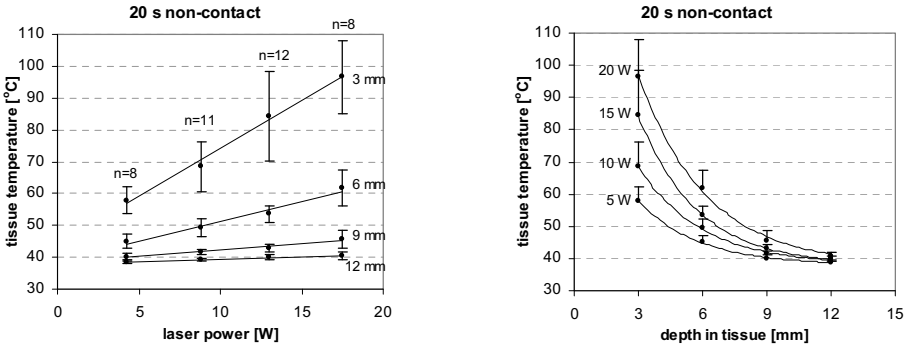


Figure 3: Mean measured temperatures ± sd after 20 s non-contact laser irradiation, adjusted to body temperature (38°C). Linearity of temperature increase with laser power is evident (linear fits, $R^2 \geq 0.97$). Temperature decrease with depth of the tissue was fitted by an exponential equation ($T(x)=38+Ae^{B(x-C)}$, $R^2>0.99$, where 'x' represents the distance from the tissue surface and $T_0=38$°C. In the temperature versus depth figure, only +sd values are shown to improve readability of the figure, avoiding overlapping error bars.

During contact mode laser irradiation of prostate tissue samples, carbonization and subsequent vaporization of the tissue occurred directly under the fiber-tip. This resulted in the formation of a narrow (1-2 mm in diameter) char-coated crater. The depth of this crater varied linearly ($R^2=0.98$) with laser exposure time and was 1.29 ± 0.67, 2.28 ± 0.62 and 3.48 ± 0.56 mm for 5, 10 and 20 s of 10 W laser irradiation, respectively.

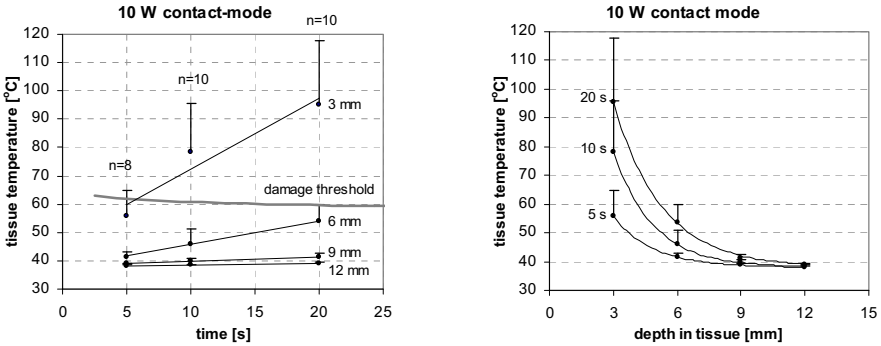


Figure 4: Measured tissue temperatures (means ± sd) after 5, 10 and 20 s of 10 W (8.8 W actual fiber output power) contact mode laser application at prostate tissue samples. Results were adjusted to body temperature. The calculated Arrhenius damage threshold (Henriques 1947) is displayed in grey. Linear fits are displayed for temperature versus time. Exponential fits trough the mean values are displayed for temperature versus tissue depth ($T(x)=38+Ae^{B(x-C)}$, $R^2>0.99$). Only +sd values are shown to improve readability of the figure.

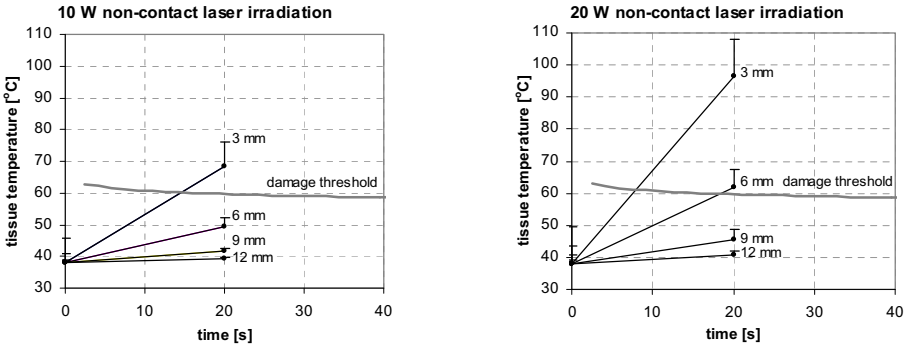


Figure 5: Reconstructed temperature increase during 20 s non-contact laser irradiation at 10 W (n=11) and 20 W (n=8), showing the temperature increase at 3, 6, 9 and 12 mm tissue-depth respectively. The grey line represents the Arrhenius irreversible damage limit for linear temperature increase in time, using A_f and E_a as determined by Henriques (1947).

Theoretical prediction of tissue damage threshold limits

Theoretical thermal damage limits were constructed by combining the Arrhenius damage integral (Eq. 1) with the tissue temperature distribution in time. An Arrhenius damage threshold graph was constructed by solving the Arrhenius equation (Eq. 1) numerically for Ω=1 and *linear* temperature increase in time: T(t)=38+s*t ('s' is the slope, varying from 0.2 to 10 $^{o}C\ s^{-1}$ as to cover the range of heating rates in the experiment; Figure 6). The resulting damage-threshold graph is displayed (grey line) in Figure 4 (contact mode) and Figure 5 (non-contact mode), demonstrating the onset of thermal damage in the measured temperature versus time distributions, according to the applied rate parameters (Henriques 1947).

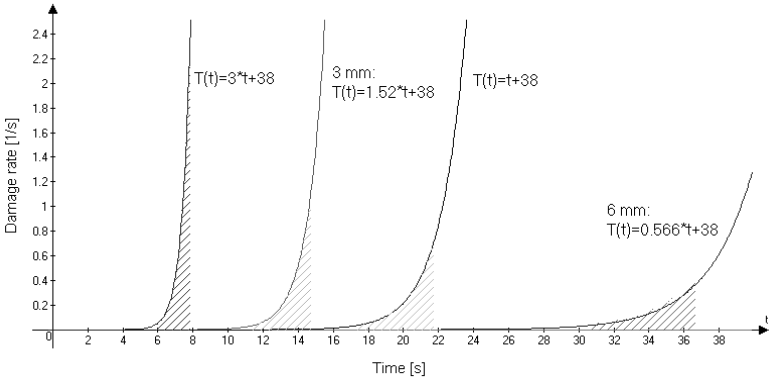


Figure 6: Example of plots of $y=Ae^{-E/RT(t)}$, for different heating regimes (T(t)) and numerical calculation of Ω = 1 (area under curve = 1) to determine τ (point of 63 % protein denaturation or irreversible tissue damage). The 3 mm and 6 mm graphs denote the temperature increase and accompanying damage threshold time (τ) at 3 mm and 6 mm tissue depth during 10 W non-contact laser exposure.

Histological evaluation and damage threshold

Depth and width of thermal coagulation damage was measured macroscopically after 20 s of non-contact laser exposure at 5, 10, 15 and 20 W. The results are displayed in Figure 7,

together with the theoretically predicted damage depth. For the 15 W setting, measurements were taken after 5, 10, 15 and 20 s, as to examine damage progression in time (Figure 8). Lesion volume was estimated by assuming a half ellipsoid: volume=$\pi/6$*(depth)(width)^2. Histological analysis of HE stained sections showed a damage area, comparable in size to the bleached area on macroscopic examination (Figure 9). A threshold temperature value for acute laser-induced thermal damage can be retrieved via several ways using the histological results (Table 1). It has to be noted that damage boundaries of 5 W exposures and of 5 s exposures were not as sharp as for the other exposure regimes. Coagulation-damage extent underneath the contact mode crater was < 1 mm. Using the threshold (T=69 ± 6^{o}C) for acute tissue damage as determined using the non-contact histology results (Table 1), the damage extent underneath the vaporizarion crater was calculated (Table 2).

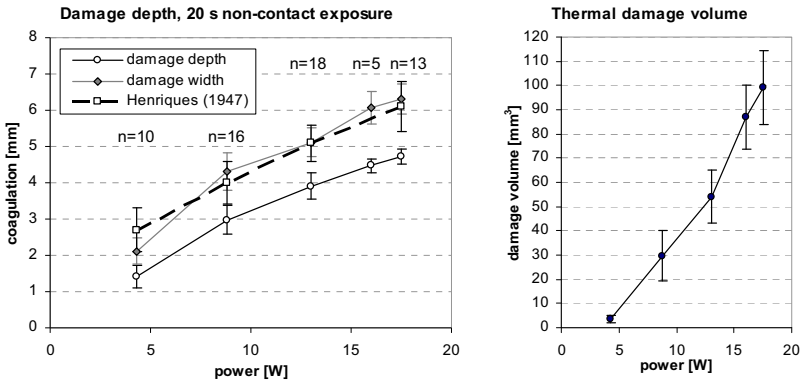


Figure 7: Macroscopically measured damage depth and width after 20 s non-contact laser exposure, means ± sd. The predicted damage depth according to Arrhenius calculation is also displayed. Damage volume was calculated assuming a half ellipsoid shape.

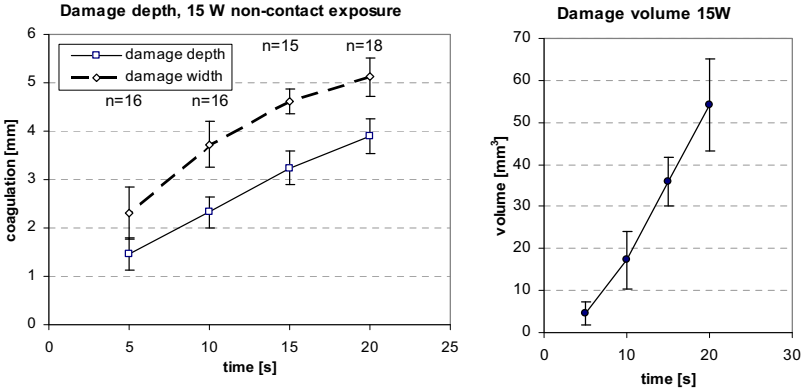
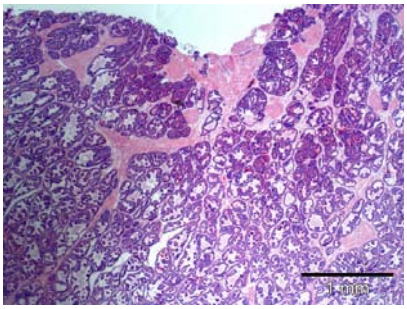
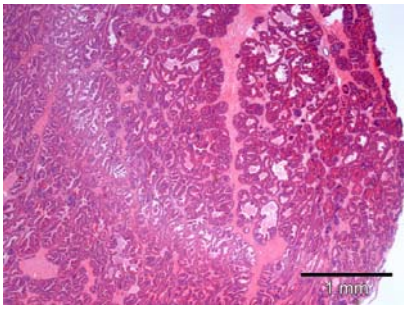


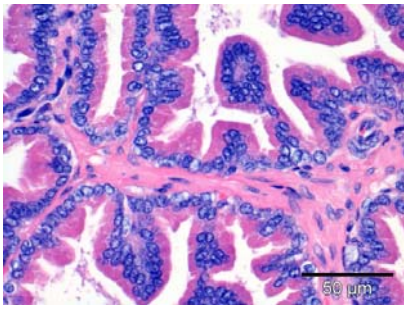
Figure 8: Macroscopically measured damage depth and width in time of 15W non-contact laser exposure, means ± sd. Damage volume was calculated assuming a half ellipsoid shape.



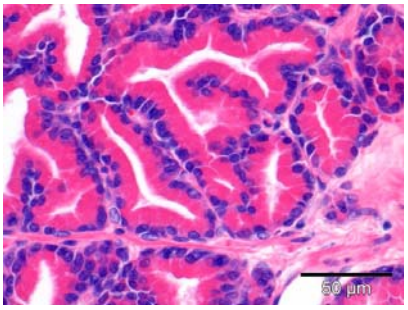
A. Thermal lesion, 15 W, 20s



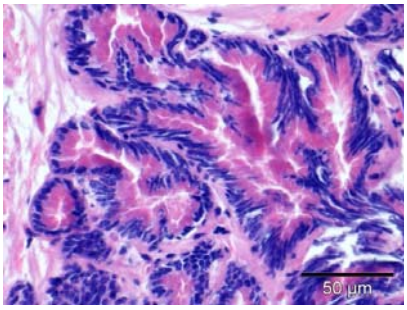
D. Thermal lesion, 20 W, 20s



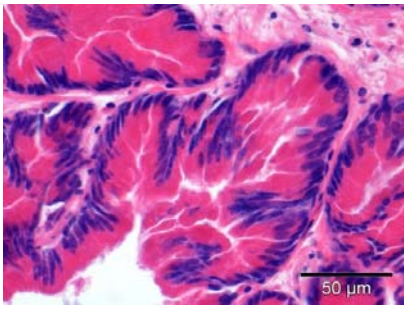
B. Normal tissue



E. Normal tissue

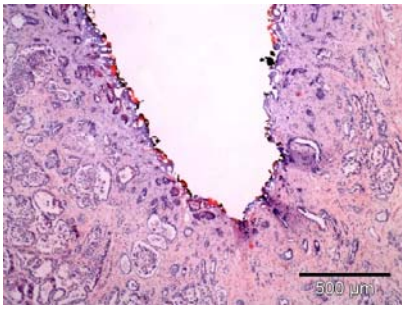


C. Thermal damage

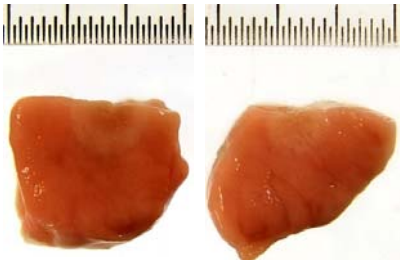


F. Thermal damage

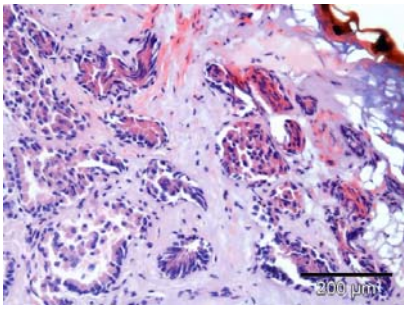
Figure 9: Pathological changes in laser irradiated prostate tissue samples. See caption on next page.



G. Vaporization crater after 5 s contact mode exposure



I. Macroscopic images of cross sections of 20s, 15 W and 20 W non-contact exposure, mm-scale included



H. Magnification of crater edge near crater floor

Figure 9, continued: Pathological changes in laser irradiated prostate tissue samples. Macroscopically, thermal damage was distinctly visible as whitening of tissue (I). Microscopic examination was performed on HE stained sections of thermal lesions. A, B, C: 20 s, 15 W non-contact exposure. D, E, F: 20 s, 20 W non-contact exposure. G, H: contact mode laser exposure. Using low magnification, HE stained sections of non-contact lesions showed increased dye uptake of an area, comparable to the macroscopic lesions (A, D). Note that the changes in dye uptake can be subtle and are often better appreciated on a macroscopic level. Tissue damage after 20 W laser exposure sometimes showed a slightly pale stained zone at the periphery of the lesion (D). At high magnification, thermal tissue damage was noticeable by increased dye uptake (eosinophilic cytoplasm and darker stained nuclei), less granulated cytoplasm, loss of nuclear detail and conformational changes of the nuclei and cells, the nuclei many times having a stretched appearance (C and F versus B and E). Contact mode laser exposure caused vaporization of tissue (G) and showed additional superficial vacuole formation, caramelization (brown-red) and carbonization (black) (H). The transition of carbonization and thermal damage to normal tissue under the vaporization-crater surface was generally <1 mm (G). Loss of birefringence was noticeable, but was not a convincing marker of thermal damage due to the limited amount of birefringence in native prostate tissue.

Table 1: Acute tissue damage threshold temperature, derived through measured damage depths and temperature distributions, means ± sd. *Apart from an overall mean threshold temperature, a mean value is also derived from only the 10W-20s and 15W-13.6s results, excluding the highest exposures, for reasons discussed in the text. n_{damage} and n_{temp}: sample sizes of damage and temperature measurements respectively.

Point of measure	n_{damage}	Damage [mm]	n_{temp}	$T_{threshold}$ [°C]	Mean $T_{threshold}$ [°C]	Mean $T_{threshold}$ [°C]*
10W, 20s	16	2.97 ± 0.39	11	69 ± 8	70 ± 4	69 ± 6
15W, 13.6 ± 1.6s	15	3.0 ± 0.36	12	70 ± 10		
15W, 20s	18	3.91 ± 0.36	12	71 ± 8		
20W, 20s	13	4.72 ± 0,20	8	72 ± 9		

Table 2: Damage depth underneath the crater floor after 5, 10 and 20 s contact mode laser exposure at 10W (sd in crater depth is included in the calculations for damage depth).

Contact mode 10 W	n	Mean crater depth [mm]	Damage depth from crater floor [mm]
5 s	8	1.29 ± 0.67	0.71 ± 0.97
10 s	10	2.28 ± 0.62	1.22 ±1.06
20 s	10	3.48 ± 0.56	0.92 ± 0.98
Mean damage depth from crater floor: 0.93 ± 0.58 mm			

Discussion

Temperature increase was measured in prostate tissue samples during contact and non-contact mode laser application. The onset of thermal damage was predicted using an Arrhenius equation, which serves as a model for *in vivo* laser induced tissue damage. Histological examination of laser irradiated tissue was used to determine the threshold temperature for acute thermal damage in *ex vivo* prostate tissue. Effects after short duration laser exposure were considered, as they reflect the situation seen during resection surgery. The cooling effect of blood flow on heat distribution is considered to increase with increasing laser exposure time (Hillegersberg 1993, Anvari *et al* 1994, Sturesson *et al* 1997). It is not always observed with short duration laser exposure (Fried *et al* 2000) and is assumed to be negligible up to 20 s of irradiation duration.

In general, thermal tissue damage is dependent on both temperature and the duration of increased temperature. Several studies mention damage threshold temperatures ranging from 43°C (with treatment-times up to 180 minutes (Nissenkorn and Meshorer 1993)) up to 60°C (treatment times less than 1 minute). Threshold temperatures in the range of 60-70°C are mentioned for *acute* (instantaneous) tissue damage in several tissues throughout the literature (Jacques and Prahl 1987, Thomsen *et al* 1989, Rem *et al* 2001). Nau *et al* (1999) recorded a rapid change in scattering coefficient with onset of coagulation between 60 and 65°C in canine prostate tissue. However, these tissue samples had a temperature history of subsequent exposures to increasing temperatures and therefore do not represent *acute* injury. Graham *et al* (1999) suggested a coagulation threshold of around 60°C for several tissues, including human prostate, using MRI techniques. However, prostate tissue showed a greater variability in results and showed prolonged MRI changes at higher temperatures compared to other tissues, leaving a definitive result unclear.

Solving the Arrhenius equation for linearly increasing temperature in time, using the rate parameters derived by Henriques (1947), returns a relatively constant damage threshold value of 60 ± 1°C for up to 30 s exposure (Figure 4 and 5). This value differs from the threshold temperature derived through histological examination (T=69 ± 6°C), which indicates that the used Arrhenius parameters are not suitable to predict acute thermal damage in prostate tissue. The Arrhenius calculation for linear temperature increase does however support the assumption that with short term exposure, tissue damage can be described by a single threshold temperature value, as proposed by others (Whelan and Wyman 1999). Using the 60 ± 1°C threshold value, calculated using the Arrhenius equation, the predicted damage depth was derived from the measured temperature distributions to be compared to the macroscopically measured damage depth in Figure 7.

Although the Arrhenius damage integral greatly simplifies the understanding of thermal tissue damage by assuming a single first order rate process, it has been successfully used to describe the threshold of tissue damage as a function of temperature and exposure time (Pearce and Thomsen 1995). Experimental data of the Arrhenius rate parameters (E_a and A_f) have been determined for several tissues (Bischof and He 2005), including prostate. However, there are currently no Arrhenius rate parameters that describe short-term thermal damage in prostate tissue. Arrhenius parameters were determined *in vitro* for human BPH tissue (Bhowmick *et al* 2004). The Arrhenius model was fitted to data concerning a 90% drop in viability of BPH stromal tissue, implicating a threshold of 1 minute at 70°C. Skinner *et al* (2000) fitted the rate of change of optical parameters (scattering and absorption) of rat prostate at several temperatures to an Arrhenius equation. The resulting damage-time relation would imply coagulation damage at 80°C after 100 s, or at 60°C after 10 minutes. However, a mean calibration time (duration for tissue samples to acquire static temperature) of 90 s was disregarded in their methodology, which could have affected their results. Experiments, using *in vitro* rat prostate cancer cells (Dunning AT-1), gave varying results for E_a and A_f, strongly depending on methods used and on temperature range (He *et al* 2004). Most probably, different cell-proteins show conformational changes at different rates and at different temperatures and this can not be described by a single first order rate process. It seems that apart from tissue specificity, Arrhenius rate parameter values strongly depend on methodology of investigation and temperature range (Bischof and He 2005). Therefore, it seems reasonable to conclude that Arrhenius parameters can not be extrapolated outside the temperature and exposure range in which they were experimentally determined. This means that the abovementioned prostate data are not useful for a model of acute thermal damage in the present study, since short duration exposures (up to 20s) were not considered in these studies. The very limited amount of published data concerning Arrhenius relations in short exposure thermal damage is not surprising, since several experimental limitations exist using the common method of relating damage to exposure time at static temperatures, most importantly achieving an instantaneous elevated static tissue temperature, and determining the correct end-point at high temperature (Diller and Pearce 1999).

The linear reconstruction of temperature-time curves is based on a study by Manns *et al* (1998). However, at 3 mm depth with 15 W and 20 W laser powers, the recorded

temperature-graphs occasionally were more concave-shaped. This is probably caused by superficial tissue alterations (change in optical and thermal properties) induced with these power densities at the surface (Nau *et al* 1999, Skinner *et al* 2000, Ritz *et al* 2001). Increased scattering (and to a lesser extent decreased conduction) of coagulated tissue will change temperature distribution: superficial temperatures would increase faster, while deeper in the tissue temperatures would increase slower compared to the non-coagulated state. Another possible effect contributing to a more concave-shaped temperature rise could be increased heat diffusion with increasing temperature (i.e. high power) and exposure time, for which thermal relaxation of the tissue can no longer be neglected up to 20 s exposure. However, temperature increase at 6, 9 and 12 mm does not seem to be affected much by superficial alterations or increased heat diffusion, showing linear increase, suggesting that the effect on temperature distribution at these depths is minimal for 20 s laser exposure.

The Arrhenius-predicted damage graph in Figure 7, although using an incorrect threshold value ($60 \pm 1^{o}C$, derived from Arrhenius skin parameters (Henriques 1947)), is expected to be parallel to the measured damage graph. The decreasing trend in damage depth with increasing laser power of the measured damage compared to the predicted damage, might be caused by a slight overestimation of the temperature used for the prediction. This overestimation could have been introduced by adjusting the temperatures to $38^{o}C$ at t=0 s, disregarding some of the changes in optical-thermal properties and heat diffusion that would occur at these higher temperatures (as discussed in the previous paragraph). The effect is small and not apparent up to 10 W. However, it is probably the cause of the increasing trend in determined threshold temperature with increasing laser power and exposure time in Table 1. Therefore, the threshold temperature for acute tissue damage in the present study derived through histological examination of 10W laser exposure and 15W damage progression up to 3 mm (t=13.6 s) is considered most reliable ($T=69 \pm 6^{o}C$, Table 1), excluding highest power/exposure settings (20W/20s and 15W/20s).

Thermal tissue damage was inflicted and evaluated in *ex vivo* specimens. Our histological results comply with common observations in temperature-induced tissue damage (Pearce and Thomsen 1995). The possibility that true damage extend does not match our observations can not be ruled out, since the most reliable method for tissue damage evaluation would be to wait 3 days using living tissue (Pearce and Thomsen 1995). However, several studies indicate that our method of damage evaluation is valid for short exposure experiments. In rapid heating regimes in rabbit myocardium (up to 20 s exposure (Pearce and Thomsen 1995)) and in contact mode experiments (Janda *et al* 2002) the transition of coagulated to normal tissue was abrupt and visible macroscopically. Lethal injury that is not directly visible, or not visible *in vitro*, may occur at temperature-time regimes that do not induce coagulation but destroy enough intracellular mechanisms to kill cells. Generally, to induce a substantial zone of non-coagulative cell death, prolonged exposure times (minutes-hours) are required, which were not used in the present experiment. Several *in vivo* transurethral canine vaporization and coagulation laser prostatectomy studies, with exposure times of *up to 10 minutes*, typically created coagulation-necrosis surrounded by a distinct rim of hemorrhage representing the transition to normal tissue (Muschter and Perlmutter 1994, Perlmutter and Muschter 1994,

Motamedi *et al* 1995, Peters *et al* 2000). In time, the coagulation-necrosis zone sloughs off and a cavity remains (Gill *et al* 1994), dimensions of which do many times not exceed dimensions of direct postoperative coagulation-necrosis zone (Cromeens *et al* 1994, Cromeens *et al* 1996, Kuntzman *et al* 1996, Kuntzman *et al* 1997). Information about postoperative expansion of visible lesions in time varies, however, and is likely to be dependent on temperature history of the tissue. One study of *20 minute* exposure laser prostatectomy, using a cylindrically diffusing fiber tip, reported a broad transition zone: a non-coagulative degenerative zone of 4 - 5 mm surrounded the coagulation-necrosis zone in the acute phase, which developed coagulation necrosis en sloughing after one week (Suzuki *et al* 1994). On the other hand, acute damage (1 mm hyperemic zone included) surrounding contact mode incisions in rabbit liver *in vivo* did not extend in time (Judy *et al* 1993), supporting the assumption that *short-term* acute thermal tissue damage extent can be measured using the initial visible tissue changes.

The narrow margin from coagulation necrosis to viable tissue in short exposure regimens can be explained using the Arrhenius damage integral. Due to its exponential nature, temperature-dependent irreversible tissue damage occurs within a relatively narrow temperature range (Pearce and Thomsen 1995), which is crossed in a relatively short time interval in rapid tissue heating procedures (Figure 6). In slow-heating procedures, creating a rather gradual temperature gradient in depth of the tissue, the temperature range to overcome coagulation threshold is spread over a larger time interval and a larger area of the tissue and the transition zone becomes broader and less well defined on histology. Furthermore, a substantial zone of non-coagulative cell death and of sub-lethal cell injury can occur due to prolonged supra-physiological temperatures.

The less distinctly visible damage boundaries at lowest power and at shortest exposure time are probably caused by the fact that Nd:YAG laser-induced tissue damage does not start at the tissue surface to travel inwards the tissue, showing a clear “damage-front” at all times. Instead, the 1064 nm irradiation propagates inside the tissue and spreads over a certain volume through scattering before being absorbed and heating takes place. Heating is not equally distributed over this volume and coagulation starts in a certain area in which the damage threshold is initially crossed (area of highest temperature). Initial damage threshold is probably distributed over a gradual transition, with accompanying vague margins.

It was expected that in contact mode, temperature and damage distributions underneath the surface are reduced in depth compared to non-contact mode. A more pronounced decrease of temperature in depth of the tissue with contact mode, caused by absorption of laser energy by the carbonized tissue surface, compared to non-contact mode was not directly evident in the study reported here, because of the expanding vaporization crater underneath the laser fiber (which was fixed in position). Temperature distributions measured during these contact mode experiments are not readily valid to reflect contact mode temperature distributions during surgery because movement of the fiber tip prevents crater formation in depth of the tissue. Therefore, shorter laser times (5 and 10 s) represent a more realistic contact mode laser model. However, damage progression underneath the contact/vaporization mode laser

resection surface (bottom of the crater), as determined using a damage threshold of T=69 ± 6^{o}C, was 0.9 ± 0.6 mm in the experiments reported here (Table 2). This was consistent with histological findings (Figure 9).

Conclusions

It is concluded that thermal damage is governed by a constant threshold temperature during short exposure (< 30 s). Under this assumption, threshold temperature is not coupled to duration in short term heating. An acute damage threshold temperature was determined *in vitro* for canine prostate tissue (69 ± 6 ^{o}C). The extent of tissue damage during contact mode surgery will generally be 0.9 ± 0.6 mm or less underneath the surface (Table 2). The free beam/non-contact technique can produce deeper thermal coagulation, at a rate described in Figures 7 and 8.

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CHAPTER 7

Partial Prostatectomy using a Nd:YAG laser for Management of Canine Prostate Carcinoma

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Abstract

Objective – To report a technique for partial prostatectomy by laser dissection and to evaluate outcome and complications in dogs with prostate carcinoma (PCA).

Study design- Experimental and clinical case series.

Animals – Four normal dogs and 8 dogs with PCA.

Methods – Subcapsular partial prostatectomy, sparing the urethra and the dorsal aspect of the prostatic capsule, using Nd-YAG laser dissection to remove the prostatic parenchyma and control hemorrhage was performed in 4 normal dogs and subsequently in 8 dogs with histologically confirmed PCA. Additional treatment of PCA dogs included local application of interleukin-2 and systemic administration of meloxicam. Prostate size, complications, and survival time were recorded. Laser associated thermal damage to surrounding tissue was evaluated by histology.

Results – In normal dogs, no damage to the dorsal prostatic capsule or urethra was detected. In PCA dogs, median survival was 103 days (range, 5-239 days). Three dogs died from complications within 16 days, whereas 5 (median survival, 183 days; range, 91-239 days) had improvement or resolution of clinical signs. Urinary incontinence did not occur.

Conclusion – Laser assisted subcapsular partial prostatectomy can be performed in dogs with PCA without development of postoperative incontinence.

Clinical relevance – Subcapsular partial prostatectomy is a potential palliative treatment for PCA in dogs and may lead to the resolution of clinical signs for several months.

Introduction

Canine prostate carcinoma (PCA) is uncommon with an estimated prevalence of 0.2 to 0.6%.[1] True prevalence is unknown as population-based data is not available.[2] Canine PCA has an invasive growth pattern and commonly metastasizes to the sublumbar lymph nodes; occasionally, metastases to the lungs and lumbar vertebrae are observed.[3] Castration has no effect on disease progression, nor does it prevent occurrence of PCA; in fact, it appears that castrated males are at an increased risk of developing PCA compared with intact males.[4] Clinically, canine PCA therefore resembles late stage, hormone-independent human PCA and the dog is an appropriate model for understanding the pathogenesis of PCA in humans.

Unlike humans, total prostatectomy is not an option for treatment of PCA in dogs because of a high incidence of postoperative incontinence.[5] The cause of incontinence in dogs after total prostatectomy is uncertain. Total prostatectomy in dogs with prostatic disease is more likely to cause incontinence than prostatectomy in dogs with a normal prostate,[6,7] suggesting that primary prostatic disease rather than surgical technique may be responsible for this complication. For these reasons, therapeutic modalities for PCA in dogs need to optimize removal of neoplastic tissue without compromising urethral sphincter function, which is controlled by the hypogastric nerve lying dorsolateral to the prostate and bladder neck.[8] Ideally, the technique for removal of prostatic tissue should permit careful dissection of prostatic parenchyma and optimal control of hemorrhage to maintain good visibility and a high level of precision for maximal removal of neoplastic tissue, but preserving the neurovascular structures on the dorsolateral aspect of the prostate. We hypothesized that these principles could be respected by use of Neodymium:Yttrium Aluminum Garnet (Nd-YAG) laser to perform subcapsular partial prostatectomy to substantially reduce prostatic volume, alleviate clinical signs of PCA and maintain urinary continence.

Because removal of neoplastic tissue would be incomplete, adjuvant therapy is required to ensure that remaining neoplastic tissue and metastases are prevented from proliferating. Interleukin-2 (IL-2), a cytokine with a wide range of immunologic effects including the activation of cytotoxic T lymphocytes, natural killer cells and lymphokine-activated killer cells,[9,10] has been used systemically and intralesionally for treatment of various neoplasia in cows, horses, and humans, and reportedly induces regression of metastatic tumors in humans.[9-11] IL-2 has been administered intralesionally in doses ranging from 200,000 IU/tumor[12] to 6 million IU.[11] In addition, prostate cancer cells like other cancer cell types express cyclooxygenase-2 (COX-2), whereas normal prostatic cells do not. Although the exact significance of COX-2 for carcinogenesis is not entirely understood,[13] studies using cell cultures and in vivo mouse models show that inhibition of COX-2 could be beneficial in management of patients with PCA.[14,15] Meloxicam is a non-steroidal anti-inflammatory drug with high specificity for COX-2 inhibition[16] and is registered for long term treatment of dogs.

Thus, we report use of a Nd-YAG laser assisted technique for partial prostatectomy in 4 normal dogs, and then outcome in 8 dogs with histologically confirmed PCA also treated with intralesional IL-2 and systemic meloxicam.

Materials and Methods

Dogs

The technique was first tested in 4 non-survival, healthy, intact, adult male Beagles (weight, 14.5 - 21.5 kg; age, 5 - 7 years), then used in 8 patients with a suspicion of PCA, based on cytological examination of an ultrasound-guided fine needle aspirate of the prostate.

Anesthesia

Dogs were premedicated with medetomidine (20 μg/kg intravenously [IV] initially then 10μg/kg hourly), and anesthesia was induced with propofol (1-2 mg/kg IV to effect) and maintained with isoflurane (< 1% end-tidal concentration in 50% air and 50% O_2). Lactated Ringers solution (5 mL/kg/hr) was administered throughout anesthesia. A combination of buprenorphine (20 μg/kg subcutaneously 4 times daily until hospital discharge) and meloxicam (0.2 mg/kg before surgery, then 0.1 mg/kg orally once daily) was administered for analgesia.

Surgical procedure

We performed subcapsular partial prostatectomy, sparing the urethra and the dorsal aspect of the prostatic capsule including the neurovascular structures essential to the normal function of the urethral sphincter. A urethral catheter was inserted to allow localization of the urethra during surgery. The prostate was approached by caudal median celiotomy, the bladder was retracted cranially, and periprostatic fat tissue was dissected from the prostate to allow observation of the ventral portion of the prostatic capsule. A Nd-YAG surgical laser (Medilas 40 N, MBB-Medizintechnik GmbH, München, Germany) with a 600 μm optical fiber (Ultraline, Heraeus LaserSonics, Milpitas, CA) was used at 10 W (continuous wave) to incise the ventral part of the prostatic capsule along the midline. Prostatic tissue was bluntly separated from the capsule, then parenchymal segments were removed using laser dissection to control hemorrhage. Prostatic tissue samples were submitted for microscopic examination to confirm a diagnosis of PCA. The urethra remained intact and prostatic tissue was removed on each side of the urethra as far dorsally as possible. Finally the edges of the capsule were trimmed and the capsule was sutured ventrally over the urethra without leaving dead space, using a continuous pattern of 3-0 polyglecaprone 25, then the celiotomy was closed in layers.

Postoperative evaluation

Prostate size was measured pre- and postoperatively by ultrasonography and prostate volume calculated.[17] Prostate volume was divided by body weight to obtain prostatic index to compare prostate volume in dogs of differing body size. Experimental dogs were euthanatized immediately postoperatively and the prostate was submitted for histologic examination to determine laser thermal damage to the dorsal part of the prostatic capsule and urethra.

Prostatic tissue was fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin.

Postoperative treatment

After surgery dogs with PCA, IL-2 (4.5 million IU in 1 mL 0.9% NaCl) was injected into the remaining prostatic tissue and meloxicam (0.1 mg/kg orally once daily) was administered. Dogs were examined 1 month postoperatively, then every other month. On each follow-up visit, chest radiographs were taken and abdominal ultrasonography performed.

Statistical analysis

Survival times were reported as median and range. Prostate dimensions were as mean ± SD. Where appropriate, a t-test for paired samples was performed to compare preoperative and postoperative means. Pearson's correlation was used to test the correlation between prostate index and survival. Significance was $P < .05$ (2-tailed).

Results

In normal and PCA dogs, use of the Nd-YAG laser provided excellent control of hemorrhage during prostatic tissue dissection. Occasional hemorrhage from larger vessels running beneath the prostatic capsule was controlled by laser or electrocautery. In normal dogs, prostatic volume was reduced by 50.5 ± 15.0% ($P = .013$) and ~2 mm of periurethral tissue remained. There was no visible damage to the dorsal prostatic capsule or urethra on histologic examination.

Dogs with PCA

Clinical signs associated with PCA were present for 1-4 months except for 1 dog that had urethral blood loss for >36 months (Table 1). Median survival time was 103 days (range, 5 - 239 days (Fig 1). There was a weak positive, but non-significant correlation ($R^2 = .32$, $P = .56$) between prostatic index and survival time (Fig 2). One dog was omitted from this analysis because the prostate contained large fluid filled cysts and the measured volume of the prostate did not accurately reflect the volume of neoplastic tissue. Five dogs recovered well from surgery and clinical signs improved or resolved; median survival time was 183 days (range, 91 - 239 days). None of the dogs developed urinary incontinence.

Table 1. Signalment, clinical signs and survival of 8 castrated male dogs with prosate carcinoma treated by partial prostatectomy facilitated by Nd:YAG laser dissecction

Breed	Age (years)	Clincal Signs	Prostate Index[a]	Survival (days)
Bearded Collie[b]	11	Tenesmus, haemorrhagic urethral discharge	0.34	183
Labrador Retriever	10	Purulent urethral discharge	0.82	201
Heidwachtel	10	Tenesmus, stranguria	0.69	114
Howawart	10	Pollakiuria, haemorrhagic urethral discharge	0.28	91
Bouvier des Flandres Crossbreed	14	Tenesmus, haematuria, haemorrhagic urethral discharge	n/a[d]	6
Bearded Collie	11	Tenesmus, dysuria, pollakiuria	0.74	239
Golden Retriever	11	Severe stranguria[c]	0.49	16
Labrador Retriever[b]	9	Tenesmus, severe stranguria[c]	0.36	5

[a]Volume of prostate (mL) divided by body weight (kg)
[b]Patients diagnosed with metastases to the local lymph nodes.
[c]Severe stranguria was defined as stranguria requiring catheterization.
[d]Prostate contained large cysts, therefore prostate size was not considered a relevant indicator of the volume of neoplastic tissue

All dogs were eventually euthanatized because of recurrence of clinical signs like dyschezia and dysuria. In 1 dog, these signs were related to urinary tract infection that resolved with antibiotic administration. Clinical signs in all dogs recurred even though the prostate was not clinically substantially enlarged compared with its immediate postoperative size. Necropsy examination of the prostate invariably revealed an aggressive histologic pattern with invasion of tumor cells into blood vessels and the prostatic capsule.

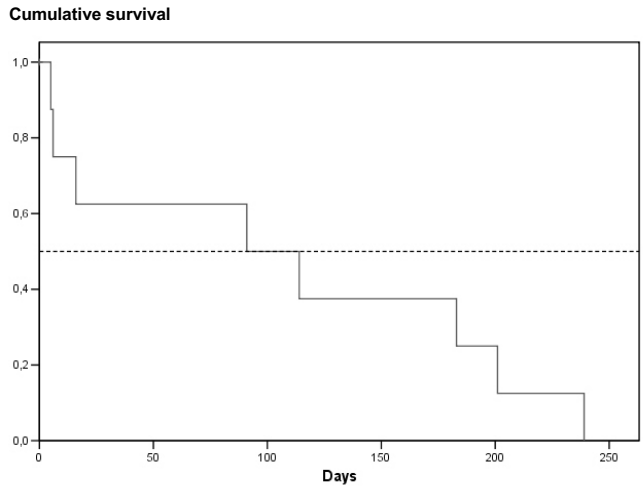

Fig 1. Kaplan-Meier cumulative survival curve for 8 dogs with prostate carcinoma (PCA) treated by partial prostatectomy facilitated by use of Nd-YAG laser dissection.

Three dogs developed postoperative complications and died or were euthanatized within 16 days of surgery. In 1 dog, severe dysuria present before surgery did not resolve and the dog was unable to urinate postoperatively, despite administration of a sympathicolytic drug (prazosin, 0.033 mg/kg orally every 8 hours) and meloxicam. Another dog was euthanatized because of bilateral ureteral obstruction from tumor ingrowth into the trigone region of the bladder. Both dogs were admitted preoperatively with severe stranguria requiring daily catheterization. The 3rd dog, a Bouvier des Flandres mixed breed that had clinical signs of prostatic disease for 36 months before admission had 2 very large prostatic cysts that were drained and omentalized before subcapsular prostatectomy. Preoperatively, there was moderate hypoalbuminemia, slight thrombocytopenia, and slightly increased activated partial thromboplastin time (APTT). Intraoperatively, the dog had an increased bleeding tendency suggestive of a clotting disorder. Immediately postoperatively, the dog had signs of oliguria requiring aggressive fluid therapy including plasma transfusion. On the day after surgery the dog developed severe hind limb edema.

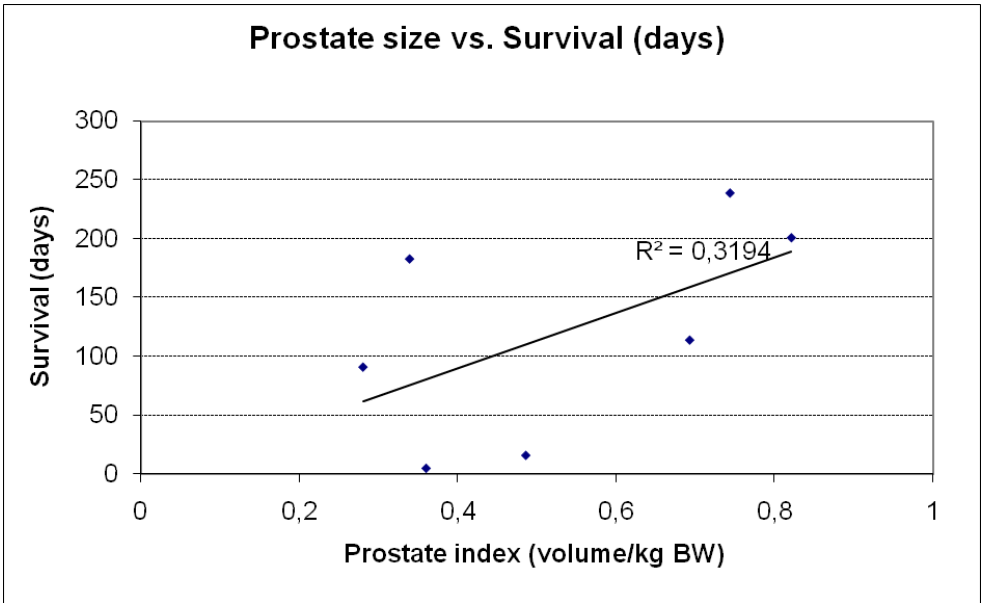

Fig 2. Linear regression curve showing survival as a function of prostatic index (prostate volume [mL] divided by body weight [kg])

Ultrasonographic examination of the caudal abdomen revealed a dorsally enlarged prostate, possibly from a hematoma, however no evidence of abnormal circulation to the hind limbs was detected. It was not clear whether the edema was caused by hypoalbuminemia, if changes in the prostate region caused disturbance of lymphatic drainage from the hind limbs, or whether it was a reaction to the plasma transfusion. Despite continued fluid therapy, blood transfusion, and medical management the dog did not recover appropriately. The owners insisted on taking the dog home and it died 6 days after surgery.

Two 8 dogs had metastases to the sublumbar lymph nodes at the time of surgery. A 3rd dog developed visible pulmonary metastases during follow-up (2 metastases were visible on thoracic radiographs 1 month postoperatively and remained visible during subsequent rechecks).

Discussion

In normal dogs, the technique we report was effective in removing prostate parenchyma and controlling hemorrhage and resulted in a significant reduction in prostate volume. Ideally, these dogs should have been followed postoperatively to check for incontinence and other complications; however, that experimental design was considered ethically unacceptable in The Netherlands. The absence of histologic evidence of thermal damage to important regional anatomic structures suggests that Nd-YAG laser may safely be used to dissect prostatic tissue within millimeters of either the urethra or the dorsal prostatic capsule with its associated neurovascular structures. This assumption was confirmed by the outcome of the procedure in dogs with PCA where none developed urinary incontinence.

Various partial prostatectomy techniques have been described using either electrocoagulation,[18] ultrasonic aspiration[19,20] or Nd-YAG laser excision[21] in normal dogs or dogs with benign prostatic disease. Subcapsular dissection as we describe was similar to previously reported techniques using electrocoagulation[18] and ultrasonic aspiration.[19,20] The absence of postoperative incontinence in our dogs corresponds to similar results reported for other dissection techniques. Another study reported partial prostatectomy using a Nd-YAG laser[21] not for subcapsular dissection of prostate tissue but resection of prostatic capsule and parenchyma on each side of the urethra, including the dorsolateral aspect of the capsule. With that technique no incontinence occurred in normal dogs but the postoperative incidence in dogs with prostatic disease was similar to the incidence after total prostatectomy. Our results corroborate our hypothesis that partial prostatectomy maintaining the dorsolateral aspect of the prostatic capsule intact is seemingly necessary to avoid postoperative incontinence.

Canine PCA is an invasive tumor and complete removal of the prostate to obtain sufficient tumor margins leads to intractable and thus, unacceptable urinary incontinence. Marginal, partial prostatectomy without additional radiotherapy is, by definition, a palliative procedure. In most of our dogs clinical signs improved substantially or resolved completely. Dogs survived up to 240 days after surgery before clinical signs recurred and the dogs were euthanatized. Interestingly, clinical signs recurred in the absence of substantial prostatic enlargement. The invasive nature of PCA seen at necropsy indicated that recurrence of signs was probably because of tumor progression into the lumen of the urethra or the wall of the rectum.

Published information on outcome after diagnosis of PCA in dogs is very sparse. In 1 report describing clinical aspects of PCA in dogs without surgical treatment, 58 of 72 dogs were euthanatized at diagnosis and mean survival time for those surviving > 1 week was 30

days. Reports of surgical treatment of PCA in dogs is limited to a few cases. Surgical placement of a retained urethral catheter in 3 dogs with PCA and stranguria enabled the dogs to survive 3 - 5 months after surgery.[22] In another report, 3 male dogs with prostatic neoplasia were treated by transurethral resection using an electrocautery loop (combined with intraoperative radiation therapy in 2 dogs).[23] Survival times were 32, 74, and 264 days; however, 2 dogs were diagnosed with prostatic transitional cell carcinoma and 1 with undifferentiated carcinoma, therefore it is uncertain whether these results are comparable to ur patients. Treatment of PCA by transurethral photodynamic therapy allowed 1 dog to survive nearly 9 months after treatment.[24] Survival times of the dogs in our study therefore compare favorably with previously published results of surgical treatment of PCA in dogs. It is possible that other forms of adjuvant treatment directed toward local control of remaining neoplastic tissue could allow prolonged survival times after laser surgery in dogs with PCA.

Severe complications developed in 3 dogs. Because of the high level of emotional distress of owners it was not possible to collect necropsy information. In 2 dogs, complications were associated with persistence of dysuria and stranguria. Because these 2 dogs had severe stranguria before surgery, we believe these complications likely resulted from tumor ingrowth into the urethra, rather than from a direct effect of the surgical procedure on the proximal urethra. In the 3rd dog, the complications were systemic (hypoalbuminemia, shock, hind limb edema) and not specifically related to the prostate. These complications probably resulted from the clotting disorder diagnosed preoperatively, however a treatment-related effect cannot entirely be excluded. Severe stranguria preoperatively (ie, stranguria requiring catheterization) may be a negative prognostic factor for survival after treatment, as evident in these dogs. Duration of clinical signs before diagnosis, tumor size at admission, and metastases to the sublumbar lymph nodes did not significantly correlate with survival time.

We lack a control group. Inclusion of a control group (dogs that did not have surgery) however, was considered a bias because these dogs often have the worst clinical signs. Additionally, owner motivation of a dog that has had surgery cannot be compared with that of an owner of an unoperated dog. Owner motivation to pursue treatment is an aspect of prime importance influencing the choice of time for euthanasia and therefore survival time after beginning treatment. Based on our experiences with these PCA dogs, we believe that partial prostatectomy facilitated by Nd-YAG laser dissection and accompanied by adjuvant treatment can be considered as a palliative treatment that can provide resolution of clinical signs for at least several months postoperatively.

Acknowledgement

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APPENDIX TO CHAPTER 7

Histological evaluation of prostate tissue damage of *in vivo* subcapsular laser prostatectomy in the normal dog

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Histology

Chapter 7 described a subcapsular partial prostatectomy in dogs as part of a treatment strategy for prostate carcinoma in dogs. Pieces of excised prostate tissue and the entire remaining prostate of mongrel dogs in this study were gathered postoperatively and stored in 4% buffered formaldehyde to be embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) for microscopic evaluation. Images if prostate tissue sections are displayed in Figure 1. The acute thermal damage at the margins of laser incisions is evident as a distinct coagulation necrosis zone, containing moderate interstitial hemorrhage. This tissue damage typically extends 0.3-0.8 mm, and occasional extent up to 1.5 mm is noted. From the histological evaluation of the several sections it was concluded that thermal damage margins remained <2.0 mm. Furthermore, the urethra was intact in all sections.

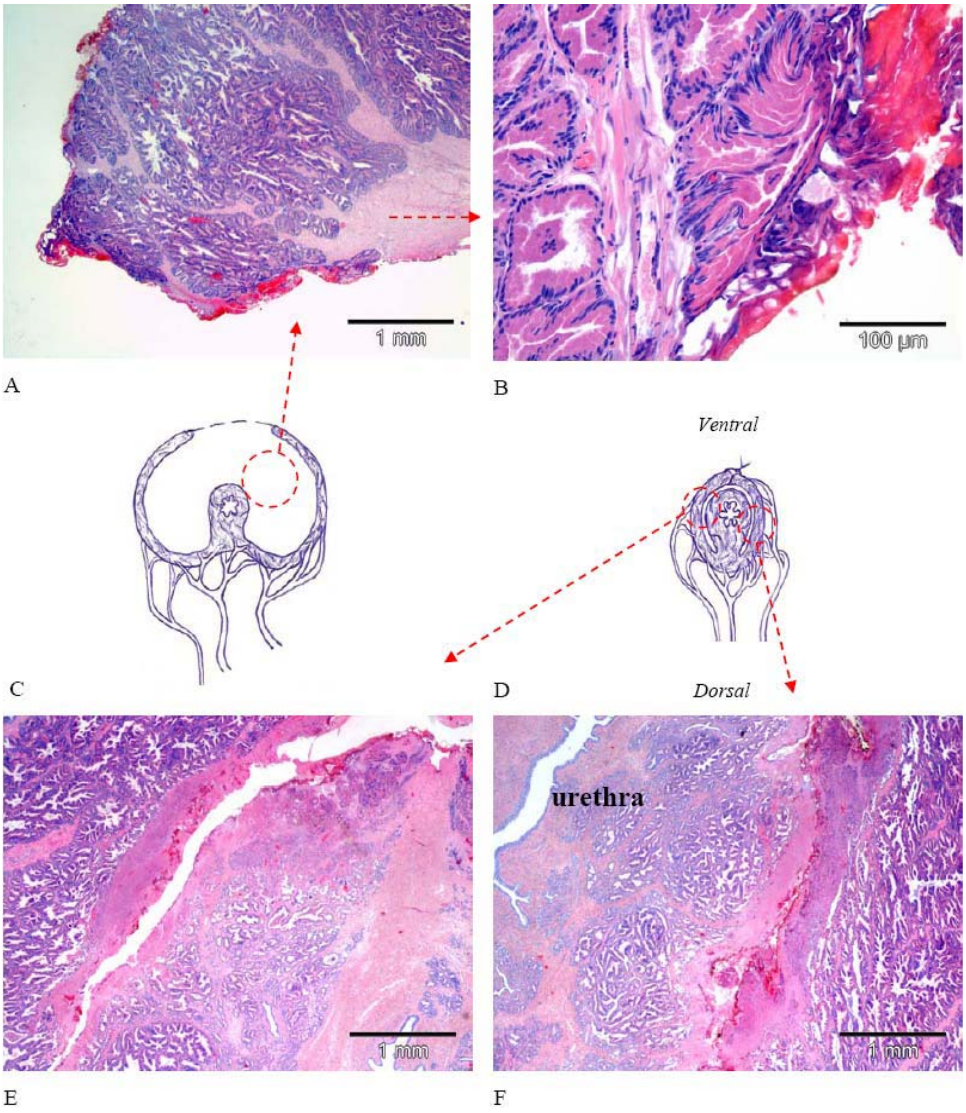

Figure 1: HE stained histological sections, showing acute thermal damage of *in vivo* Nd:YAG laser-excised prostate tissue (A and B) and the remaining prostate (E and F) after subcapsular laser prostatectomy as described in Chapter 7. During surgery, prostate parenchyma is resected leaving the capsule and urethra intact (C). Excess of ventral capsule is removed and the capsule is sutured around the urethra (D). Carbonized/caramelized tissue stains red. Thermal damage is denoted by increased dye uptake, loss of intracellular and intranuclear structure, and conformational changes of cells and nuclei. Figure B shows the transition from carbonization and conformational cell-changes to normal tissue at the margin of laser-excised tissue from figure A. In the remaining prostate, a rim of distinct coagulation necrosis is evident at the incision margins, with moderate interstitial hemorrhage (E and F). Thermal damage at the edges of laser incisions is typically <1.0 mm (range ~0.3-0.8 mm) and occasionally extends up to 1.5 mm in these sections (E), and was overall <2.0 mm. The urethra is intact.

Conclusion

These findings of *in vivo* laser surgery of the canine prostate are supported by the results of *in vitro* acute thermal tissue-damage investigation in *Chapter 6*. Furthermore, patients in the prostatectomy study were continent postoperatively.
It is concluded that the Nd:YAG laser is capable of relatively precise prostate tissue resection with acceptable accompanying thermal damage margins and can therefore be used for canine subcapsular partial prostatectomy, leaving prostate capsule and urethra intact.

CHAPTER 8

Preliminary results of intraoperative photodynamic therapy with 5-aminolevulinic acid in dogs with prostate carcinoma

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Abstract

Six client-owned dogs with prostate carcinoma were treated with a combination of partial subcapsular prostatectomy using an Nd:YAG laser, intraoperative photodynamic therapy using a halogen broad band lamp after local administration of a photosensitiser, and systemic treatment with meloxicam. Median survival time was 41 days (range 10-68 days), which compared negatively with previous reports of both (1) subtotal laser prostatectomy combined with topical interleukin-2 administration and (2) photodynamic therapy alone. Despite treatment, the disease progressed locally, causing signs of stranguria to recur, and in the form of distant metastases. The recurrence of clinical signs due to the primary tumour despite photodynamic therapy is probably largely explained by insufficient penetration of light into the tissue. Better results may be obtained using other light sources (e.g. laser) and alternative techniques of light delivery, such as fibres or catheters allowing interstitial diffusion of light.

Introduction

Photodynamic therapy (PDT) involves the topical or systemic administration of a photosensitiser followed by the delivery of light to the target area. The interaction of light with the intracellular photosensitiser causes the release of oxygen radicals leading to cell death (Moore et al., 1997). The relative selectivity of the technique lies in the topical administration to or the selective uptake by neoplastic tissue, as well as in the localised delivery of light (Moore et al., 1997).

One of the best studied photosensitisers is 5-aminolevulinic acid (ALA). ALA has been used successfully for both photodiagnosis (Baumgartner et al., 1996) and photodynamic treatment (Kennedy et al., 1996; Kennedy et al., 1990) of neoplastic lesions. ALA itself is not a photosensitiser but it induces the formation of protoporphyrin IX and other photoactive porphyrins (PAP) (Peng et al., 1997). Ester derivatives of ALA have the advantage of being more lipophilic than ALA allowing them to penetrate better through lipid membranes and into tissues (Casas and Batlle, 2002; Marti et al., 1999). ALA esters do not increase the level of PAP formed in the tissue compared to ALA, but increase the confinement of PAP to the location of the administration making them best adapted to topical rather than systemic administration (Perotti et al., 2002, , 2003).

The first experiences with the use of PDT as a treatment modality for neoplasia in companion animals were obtained in the 1980s (Cheli et al., 1984; Cheli et al., 1987). These initial promising results led to growing interest in the technique and it has been used to treat many different types of canine and feline tumours including skin tumours such as intraoral and cutaneous squamous cell carcinoma (SCC) (Frimberger et al., 1998; Roberts et al., 1991), mast cell tumours, mixed mammary gland tumours (Roberts et al., 1991), and haemangiopericytomas (McCaw et al., 2001). Other reports have presented cases of oesophageal SCC (Jacobs and Rosen, 2000) and prostate carcinoma (Lucroy et al., 2003) treated with PDT.

Canine prostate carcinoma (PCA) is a rare but consistently aggressive tumour that frequently metastasises (Cooley and Waters, 2001). In humans, PCA is mostly treated by androgen ablation or radical prostatectomy (Damber, 2005; Damber and Khatami, 2005), but PCA in dogs requires other forms of treatment. Canine PCA does not respond to androgen ablation. In fact, PCA occurs more commonly in castrated males (Teske et al., 2002). In addition, radical prostatectomy is complicated by a high prevalence of incontinence (Goldsmid and Bellenger, 1991).

Subtotal intracapsular prostatectomy can relieve clinical signs without causing incontinence (L'Eplattenier et al., 2006), however since tumour removal is incomplete with this technique, local control of the neoplastic tissue remaining around the urethra and in the dorsal portion of the prostate is critical to prevent recurrence of clinical signs and to increase survival times. Recently, partial prostatectomy using Nd:YAG laser dissection was combined

with local injections of interleukin (IL)-2 and postoperative systemic treatment with the cyclo-oxygenase (COX)-2 inhibitor meloxicam to manage PCA in eight dogs (L'Eplattenier et al., 2006). The dogs survived up to 8 months postoperatively (median 103 days, range 5-239 days) and most were eventually euthanased because of recurring clinical signs attributed to the primary tumour (most often stranguria).

In another report, one dog with PCA was successfully treated with PDT alone, and survived for 9 months after treatment (Lucroy et al., 2003), suggesting PDT may be an effective way of locally controlling PCA cell growth and may be a useful adjunct to surgical debulking of the tumour.

This present study reports results of intraoperative PDT using topically administered hexyl aminolevulinate, following partial subcapsular prostatectomy in six dogs. We hypothesised that PDT would be at least as effective as local administration of IL-2 in controlling PCA locally and preventing recurrence of clinical signs after subtotal prostatectomy.

Materials and methods

Animals and preoperative assessment

Six client-owned dogs were included in the study. Inclusion criteria were echographic signs of prostatomegaly, including abnormal structure and shape of the prostate, with or without enlargement of the sublumbar lymph nodes, cytologically-confirmed diagnosis of prostate carcinoma, and the owner's informed consent. Regional lymph nodes were not assessed cytologically before surgery. Patients were excluded from the study if radiographic and/or computed tomography (CT) evaluation of the lungs and caudal axial skeleton (lumbar spine, sacrum and pelvis) revealed changes consistent with the presence of metastases.

Anaesthesia and pain management

All dogs were premedicated using medetomidine (Domitor, Pfizer, 20 µg/kg IV, redosed at half the dose every hour), then anaesthesia was induced using propofol (PropoVet, Abbott, 1-2 mg/kg IV, to effect). The dogs were intubated and anaesthesia was maintained with isoflurane (IsoFlo, Abbott, <1% end-tidal concentration in 50% air and 50% O_2). The dogs were given an infusion of Ringer's lactate (Braun Melsungen AG, 5 mL/kg per h). Postoperative analgesia was provided by a combination of buprenorphine (Temgesic, Schering-Plough, 20 µg/kg SC four times daily until the animals were released from the clinic) and the non-steroidal anti-inflammatory drug (NSAID) meloxicam (Metacam, Boehringer Ingelheim, 0.2 mg/kg PO once on the day of surgery, then 0.1 mg/kg PO once daily for the rest of the dog's life).

Surgical technique

All animals underwent a surgical procedure as described previously (L'Eplattenier et al., 2006). The technique involved a subcapsular partial prostatectomy, sparing the urethra

and the dorsal aspect of the prostate capsule including the neurovascular structures essential to the normal function of the urethral sphincter. Preoperatively, a urethral catheter was placed to allow localisation of the urethra during the procedure. Antibiotic prophylaxis was provided by administering 20 mg/kg amoxicillin and clavulinic acid intravenously at induction of anaesthesia.

The prostate was approached via a caudal midline coeliotomy. The bladder was retracted cranially and periprostatic fat was dissected from the prostate to allow visualisation of the ventral portion of the prostate capsule. Using a continuous wave Nd:YAG laser (Medilas 40 N, MBB-Medizintechnik) with a 600 µm optical fibre (Ultraline, Heraeus LaserSonics) the ventral part of the prostatic capsule was incised along the midline. Prostate tissue was bluntly separated from the capsule, then segments of parenchyma were removed using laser dissection to control haemorrhage. Samples of prostate tissue were submitted for histopathological exam to confirm the preoperative cytological diagnosis of PCA. The urethra was left intact and prostate tissue was removed on each side of the urethra as far dorsally as possible.

Photodynamic therapy

The photosensitiser hexyl aminolevulinate hydrochloride and the halogen PDT lamp (CureLight BroadBand) were supplied by PhotoCure ASA, Oslo, Norway. After the subtotal prostatectomy, 10 mL of the reconstituted photosensitiser (8 mM) were administered to the prostate by injection into the remaining periurethral and subcapsular prostate tissue. The photosensitiser was left for 1 h before light delivery in order to allow for uptake and metabolism by the PCA cells. After 1 h, the PDT lamp was calibrated following the instructions of the manufacturer and red light (570-670 nm) was delivered to the prostate at a dose of 75 J/cm^2 (light intensity 200 mW/cm^2, as described in the PhotoCure, CureLight BroadBand User Manual). During light delivery, the prostate capsule was maintained open with a small Weitlaner retractor in order to facilitate optimal penetration of light into the remaining tissue around and dorsal to the urethra. After completion of light treatment, the edges of the capsule were trimmed and the capsule was sutured ventrally over the urethra without leaving any dead-space, using a continuous pattern of synthetic monofilament absorbable material (polyglecaprone 25, size 3-0, Monocryl, Johnson & Johnson). The abdomen was closed in a routine manner.

Postoperative management and follow-up

All patients were treated postoperatively with a systematic administration of meloxicam (Metacam, Boehringer Ingelheim, 0.2 mg/kg on the day of surgery, then 0.1 mg/kg PO once daily for life). They were examined 1 month postoperatively, then every other month. On each follow-up visit, chest radiographs were taken and an ultrasound examination of the abdomen was performed.

Results

The patients included five castrated and one intact male dog. The age of the dogs ranged from 6-13 years (median age 8 years 8 months). The bodyweight ranged from 8.9-42 kg (median 22.5 kg). Signalment, clinical signs and survival time after surgery are presented in Table 1.

Table 1. Signalment, clinical signs and survival of dogs included in the study

Case no.	Breed	Castration status (age in years at castration)[a]	Age at presentation (years)	Clinical signs	Duration of clinical signs	Survival time after surgery (days)	Reason for euthanasia
1	Crossbred	MC (4)	8 ½	Stranguria	4 months	68	Stranguria
2	German Shepherd	MC (5 ½)	6	Tenesmus Stranguria	6 months 2 days	11	Urethral obstruction [b]
3	Cross breed	M	13	Micturition indoors PU/PD Slight tenesmus	3 weeks 3 weeks 3 weeks	50	Stranguria, orchitis
4	Basset Fauve de Bretagne	MC (7)	12 ½	Prostate cyst Blood discharge Weight loss	6 months 6 months 6 months	10	Bladder atony
5	Malinois	MC (1)	8	Tenesmus Stranguria Depression	5 days 5 days 5 days	58	Anorexia, depression
6	Malinois	MC (1)	9	Tenesmus Anorexia Stranguria	1 week 1 week 1 day	32	Anorexia, depression

[a] M = male, MC = male castrated
[b] Displaced retained urethral catheter

Clinical signs

Stranguria and straining to defaecate were the most common clinical signs in this study and occurred in 4/6 dogs included. Other clinical signs included weight loss, anorexia, depression, haemorrhagic urethral discharge, polyuria and polydipsia. The range of duration of clinical signs was 1 day to 6 months (see Table 1). In 3/6 patients, acute onset of stranguria was the cause of presentation. In the other cases clinical signs had been present longer, in one case as long as 6 months.

Ultrasonographic findings

In 4/6 cases, the prostate appeared to have cystic lesions on ultrasound, the cyst in one case taking up most of the prostate. In all cases prostate tissue had an irregular echotexture and contained mineralisation. None of the dogs had ultrasonographic evidence of sublumbar lymphnode enlargement.

Surgical findings

In four cases, pre- or intraoperative findings warranted a modification of the surgical technique compared to the protocol described above (L'Eplattenier et al., 2006). In 2/6 dogs (cases 2 and 5), preoperative stranguria was so marked that it required catheterisation. In these two cases, it was decided to place a retained urethral catheter (Mann et al., 1992). In 3/6 cases (cases 2, 3 and 4), the prostatic urethra was inadvertently perforated during dissection of prostate tissue. In case 2, a retained catheter was placed and the urethra was therefore not sutured. In cases 3 and 4, the tear in the urethra was sutured using a simple continuous appositional suture of 4-0 polydioxanone (PDS, Johnson & Johnson). In these cases a urinary catheter was maintained in place for 72 h.

Survival times and outcome after surgery

The median survival time was 41 days (range 10-68 days). Two patients had very short survival times of 10 and 11 days, respectively (cases 4 and 2). In case 2, a retained urethral catheter was placed during prostate surgery. The tubing connector of a 5 Fr percutaneous nephrostomy tube was removed and the tube was inserted into the urethra via a small cystotomy, such that the coiled end remained in the bladder. The dog was able to urinate during the immediate postoperative period, however on the 9th day after surgery the dog developed stranguria and urethral obstruction again. Ultrasound examination of the bladder revealed that the catheter had been dislodged in a retrograde manner and was located entirely within the bladder. The owners decided against a second surgical procedure to replace the retained catheter and the dog was euthanased 11 days after surgery.

Case 4 developed clinical signs consistent with bladder atony postoperatively (bladder distension, dribbling of urine, no attempts to urinate, large volume of urine in the bladder upon catheterisation). A urinary catheter could be introduced easily, suggesting that the micturition problems were not caused by a stenosis of the urethra subsequent to the intraoperative urethral laceration. Treatment with a sympathicolytic drug (prazosine), muscle relaxant (diazepam) and a parasympathicomimetic drug (carbachol) were not effective, so the owner decided to euthanase the dog.

The four other patients had survival times of 32-68 days and were available for follow-up examination 1 month postoperatively. All had initially improved micturition and defaecation. Upon ultrasound follow-up examination the prostate had an appearance similar to the immediate postoperative situation in two patients (cases 1 and 3). In one patient a cyst could be seen (case 6) and in one patient (case 5) invasion of the prostate tumour into the ventral bladder wall was suspected. Patient 1 suddenly developed urethral obstruction 2 months postoperatively, which was also the reason for euthanasia. Patient 3 developed stranguria and straining to defaecate 3 weeks postoperatively, a resistant *E. coli* infection was diagnosed and treated, based on the antibiogram, with oral nitrofurantoin. Treatment relieved stranguria but the dog developed an orchitis and stranguria recurred 7 weeks postoperatively, this time caused by a urethral mass diagnosed during retrograde positive contrast urethrocystography. The owner did not wish any further investigations on this mass.

Patient 5 had improved micturition and defecation for 1 month postoperatively in addition to slight urinary incontinence due to the retained urinary catheter. The dog remained however generally depressed and developed serious rectal straining approximately 6 weeks after surgery. The dog was finally euthanased 58 days postoperatively, because of depression, anorexia and a rapidly deteriorating general state. Patient 6 had improved micturition and defecation for approximately 3 weeks postoperatively. After 3 weeks, the dog became anorexic and lame on the right hind limb. Radiographic examination revealed signs of metastases in the lungs and periostal new bone formation on the ilium and right femur. The dog was euthanased 32 days after surgery because of the strong suspicion of bone metastases.

Pathological findings

All patients were correctly diagnosed by cytology and histological examination of samples obtained during surgery confirmed prostate carcinoma. Four of the six patients underwent post-mortem examination. In all four, metastatic disease was confirmed histologically. Sites of metastasis included the sublumbar lymph nodes, the lungs, the abdominal wall, jejunum, mesocolon, subcutis in perineal area, liver, kidneys, and femur. Patient 5 which had a retained urinary catheter also had an ulceration of the prostatic urethral mucosa.

Discussion

The treatment was successful in relieving clinical signs such as stranguria and rectal straining, thus improving quality of life in the patients that survived the immediate postoperative period. However, the survival times of the patients in this study compare unfavourably with those of other studies using the same surgical technique with local administration of IL-2 (L'Eplattenier et al., 2006) (eight dogs, survival up to 8 months) or other treatment modalities such as placement of a retained urinary catheter alone (Mann et al., 1992) (three dogs, survival 3-5 months), transurethral resection with or without radiation therapy (Liptak et al., 2004) (three dogs, survival 32, 74 and 264 days), or photodynamic therapy alone (Lucroy et al., 2003) (one dog, survival 9 months).

For this reason, the study was prematurely discontinued as it was no longer considered ethical to subject dogs to an invasive treatment with such a short postoperative life expectancy. However, results of different studies should be compared with great caution as reports of treatments of prostate neoplasia are sparse, the patient numbers are very low and studies include patients with different pathologic entities, such as prostate carcinoma and transitional cell carcinoma of the prostate. It is therefore possible that the dogs included in this present study represent a subpopulation of patients with particularly aggressive forms of PCA. Widespread distant metastases were found in 3/4 dogs where post-mortem data are available.

This is consistent with the previously reported incidence of metastases in canine PCA (Cornell et al., 2000).

The aim of photodynamic therapy in this study was local control of the neoplasia as an adjuvant to surgical debulking. Three dogs in this study had evidence of local progression of tumour growth and invasion into the bladder neck with signs of stranguria and urethral obstruction 7-8 weeks postoperatively, whilst the other dogs were euthanased earlier for other reasons. In the absence of a control group treated with surgery alone, it is difficult to make conclusions about the effect of photodynamic therapy. However in a previous study combining the same surgical technique with local administration of IL-2 (L'Eplattenier et al., 2006) signs of stranguria recurred 3-8 months after surgery.

Possible explanations for the failure of PDT in the present study may include insufficient uptake of photosensitiser by tumour cells, insufficient metabolism of the compound to the endogenous photoactive porphyrins (PAP) or insufficient delivery of light to activate the photosensitiser. Insufficient uptake of the compound by the cells seems unlikely as local administration and infiltration into remaining neoplastic tissue around the urethra and under the prostate capsule should ensure presence of ample quantities of photosensitiser to be absorbed by the cells. Insufficient metabolism of hexyl aminolevulinate to PAP may be a more likely explanation, since such metabolism requires time and in this study photosensitiser administration was limited to 1 h, for reasons related to the length of anaesthesia. However the duration of photosensitiser administration was based on an in vitro study of the penetration of human and porcine urothelium by hexyl aminolevulinate, in which there was evidence of PAP accumulation within the cells after incubation for 1 h (Marti et al., 1999). Light delivery and tissue penetration limits the use of PDT in the management of solid tumours.

Two factors pertaining to the light are of importance in determining tissue penetration: intensity and wavelength (Moore et al., 1997). Red light is the first choice for most PDT applications, since it penetrates tissue more efficiently than light of other wave lengths (Moore et al., 1997), and it was the type of light used in this study. However, light penetration is also influenced by the optical properties of the tissue. Previous studies have shown the optical characteristics of the prostate to vary from individual to individual, and even to vary in the course of light delivery (Jankun et al., 2005; Jankun et al., 2004; Moore et al., 2005). It is therefore difficult to estimate light penetration in this present study.

Several factors may have caused insufficient light penetration into the prostate in this study: despite the fact that the prostate capsule was held open with a small retractor to allow maximal delivery of light, the prostate tissue did not represent a flat surface, therefore the dosage of light cannot have been exactly equal in all parts of the prostate. In addition, even after maximal debulking of prostate tissue, a portion of the prostate was left intact dorsally to the urethra. Without measuring light penetration it cannot be known whether sufficient light was delivered to this area of tissue in order to cause cell necrosis. Finally, despite the use of a surgical laser for the dissection of prostate tissue in order to limit haemorrhage, the presence

of a thin film of blood on the surface of the tissue subsequently to dissection may also have interfered with penetration of light into the tissue below.

Possible light sources for PDT include lasers, diode lasers (providing monochrome light) LED and conventional lamps (providing a certain spectrum of light). In this study a halogen lamp providing red light with a bandwidth of 570-670 nm at an intensity of up to 200 mW/cm^2 (PhotoCure) was used. Although light sources other than lasers have been shown to be appropriate for PDT (Reeds et al., 2004), other devices and other techniques of light delivery (transurethral or interstitial) than the one used in this study may be necessary to ensure optimal effect of PDT.

Only one dog in this study had signs of urinary incontinence after surgery. This dog had received a retained urinary catheter during surgery to relieve signs of severe stranguria. Incontinence is most likely related to the presence of the catheter (Mann et al., 1992), rather than to damage to the innervation of the bladder during surgery. The safety of this previously described surgical technique with regards to maintenance of urethral sphincter function has already been discussed elsewhere (L'Eplattenier et al., 2006). However one dog was euthanased in the immediate postoperative period (10 days postoperatively) because of evidence of bladder atony that did not respond to treatment. None of these signs were present before the surgical procedure and this was one of the dogs that had a perforation of the urethra during surgery. Therefore a direct effect of surgery or PDT cannot be totally excluded in this case.

Of the other two dogs with intraoperative urethral perforation, one developed stranguria shortly after surgery due to dislocation of the permanent catheter placed during surgery. The other dog developed clinical signs of stranguria 3 weeks after surgery. These signs were responsive to treatment with antibiotics. In these two dogs, it is therefore unlikely that the intraoperative urethra perforation had any significance for the outcome of treatment.

Conclusions

Partial subcapsular prostatectomy followed by intraoperative photodynamic therapy of the prostate using hexyl aminolevulinate and direct light delivery to the prostate from a halogen light source does not provide sufficient local control of canine PCA to ensure survival times comparable to other treatment modalities.

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CHAPTER 9

GENERAL DISCUSSION

Canine prostate carcinoma was studied in this thesis with two main objectives: to gain insight into aspects of the pathogenesis of this condition and to develop therapeutic tools to manage the condition.

The first part of this thesis, which is focussed on aspects of the pathogenesis of canine PCA, aimed at verifying whether findings relative to PCA in humans are also valid for canine PCA. In **Chapter 3**, the expression of cyclooxygenase-2 (COX-2) was characterised in canine PCA, *in vivo* as well as *in vitro*. Studies originating from the Department of Small Animal Clinical Sciences of Utrecht University prior to the ones included in this thesis had shown that although the vast majority of tumours in the canine prostate are carcinomas, there are in fact several histological types of tumour cells, sometimes even side by side in the same prostate (Lai et al., 2008b). Aside from this, it is known that canine as well as human prostate cancer, like other types of human and canine cancers express COX-2, whereas this enzyme is not expressed in normal tissue. In human prostate cancer, COX-2 expression is higher in poorly differentiated tumours and is associated with areas of local inflammation characterised by higher densities of T-lymphocytes and macrophages, as well as increased angiogenesis, suggesting inflammation may play a role in carcinogenesis (Wang et al., 2005). The results reported in this thesis show that contrary to human PCA, the expression of COX-2 in canine PCA is not associated with the degree of differentiation of tumour cells and that the presence of inflammatory infiltrates in tumour tissue correlates with a lower rather than higher expression of COX-2. These findings were confirmed *in vitro* as it was determined that the cytokines and growth factors tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and epithelial growth factor (EGF) induced COX-2 expression in non-neoplastic cells in culture, but reduced COX-2 expression in neoplastic cells. Similarities between human and canine PCA were, however, also observed as, in both species, androgen ablation is associated with a higher degree of inflammation.
The role of the signaling pathways phospatidyl-inositol-3 kinase (PI3K), protein kinase C (PKC) and extracellular signal-related kinase (ERK/MAPK) was investigated *in vitro* by incubating cultures of non-neoplastic and neoplastic canine prostate cells with growth factors and cytokines with inhibitors of each of these three pathways. The results presented in this thesis highlight differences in signaling pathways leading to the expression of COX-2 between neoplastic and non-neoplastic cells. Addition of cytokines and growth factors to the culture medium caused an increase in COX-2 expression involving the PI3K and PKC pathways in non-neoplastic cells, but only the PI3K pathway in neoplastic cells. Furthermore, inhibition of the ERK/MAPK pathway increased COX-2 expression in non-neoplastic cells but reduced the COX-2 expression of neoplastic cells. The precise role of COX-2 in oncogenesis of canine prostate carcinoma, however, remains poorly understood.
Chapter 4 focuses on the androgen receptor (AR) gene. Although canine PCA has been found to be hormone-independent (Teske et al., 2002), and the proportion of cells possessing the AR is much lower in PCA than in normal prostate cells of healthy intact males (Lai et al., 2008a), the AR is still expressed in the cytoplasm of tumour cells, indicating there should be a role for it in the pathogenesis of PCA. In humans, variations in the sequence of the AR gene appear to be correlated with the risk of developing PCA (Hsing et al., 2000). In particular, the transactivation domain of the AR gene contains polyglutamine (CAG) repeats of varying

length. A shorter CAG repeat length confers a higher risk of developing clinically significant prostate cancer in humans (Hsing et al., 2000). The canine AR gene also contains three polyglutamine repeats, two of which are polymorphic (CAG-I and CAG-III). Our findings that dogs with PCA had significantly shorter repeat lengths in CAG-I correlated well with findings in humans. The canine repeats, however, were determined to be much shorter than the ones in the human AR gene (10 to 12 instead of 11 to 31). In humans, the mechanism proposed is that longer CAG repeats are associated with lower expression of the AR through reduced transcriptional activity (Gao et al., 1996; Palazzolo et al., 2008). Whether the relatively small variation in CAG repeat seen in dogs is sufficient to cause significant differences in the transscriptional avtivity of the AR gene is unknown at this moment and further studies examining the correlation between CAG length and expression of the AR gene are necessary to further investigate this question.

Epidemiologic studies on prostate disease in dogs conducted at the Department of Small Animal Clinical Sciences of Utrecht University showed the bouvier des Flandres to be more susceptible to PCA than other breeds (Teske et al., 2002). Similar differences in prevalence of PCA between ethnic groups are seen in humans, as well. **Chapter 4** and **Chapter 5** both focussed on breed differences in dogs and attempted to examine whether arguments that have been put forward to explain these differences in people are also valid for the breed differences seen in dogs. Among the explanations proposed for humans, of particular interest were the differences found in the serum concentration of androgens and in the DNA sequence of the androgen receptor (AR) gene. Social and dietary influences (that could equally explain differences in the incidence of PCA in the human population) are not likely to be of any importance in dogs. In humans, the higher prevalence of PCA observed in certain ethnic groups of the population has been associated with a shorter length of the CAG repeats in the AR gene, but also with higher serum concentrations of androgens and sex hormone-binding protein. In the studies reported in this thesis, a population of healthy, adult, male bouvier des Flandres was compared to a control group composed of dogs of various breeds. The results reported in **Chapter 4** show that the different alleles and the different lengths of CAG-repeats occur with different frequencies depending on the breed examined. However the total length of the repeats did not seem to be significantly different in the Bouviers, compared to other breeds less likely to develop PCA.

In **Chapter 5**, the serum concentration of androgens was examined. Despite a relatively large group size, our results failed to show any significant difference in serum androgen concentrations between the bouviers and the control group. There was a tendency towards higher serum androgen concentrations in the bouviers, but the variation in the values obtained was high and statistical significance could not be reached. Sex hormone-binding globulin (SHBG) was not measured on this thesis because of the lack of available assays for canine SHBG and because the low degree of analogy between canine and human SHBG precludes the use of human SHBG assays in dogs. Further investigation of the role of SHBG in the development of canine PCA would however be very interesting. In humans, SHBG is not just a binding protein for testosterone and other steroids, it can bind to specific receptors on prostate cell membranes, causing an increase in intracellular cAMP that, in turn, can increase the activity of the AR through various mechanisms (Ding et al., 1998; Winters et al., 2001),

Activation of the AR by SHBG in the absence of androgens might be an explanation for the development and progression of PCA in castrated dogs.
Shorter CAG-I were associated with a higher risk of developing PCA (**Chapter 4**) and with a lower concentration of di-hydrotestosterone (**Chapter 5**) suggesting a possible protective role of androgens in canine PCA. This is in agreement with the fact that PCA occurs more frequently in castrated dogs than in intact males whereas in humans, androgen-ablation effectively prevents the development of PCA. The severity of PCA based on the histological grading, however, is inversely proportional to the serum androgen concentration both in dogs and humans. In addition, PCA in humans frequently becomes hormone-independent and more aggressive despite anti-androgen treatment. This indicates that the role of androgens differs between dogs and humans regarding the initiation and development of PCA but maybe not regarding the progression of PCA.

The second objective of this thesis was to develop a treatment protocol for PCA in dogs. Management of PCA is hampered by the absence of clinical signs in the early stages of this condition and the absence of laboratory tests allowing early diagnosis, by a high metastatic potential, and by the high frequency and seriousness of side effects following radical prostatectomy (Basinger et al., 1989; Cooley and Waters, 2001). An ideal management for PCA in dogs would therefore need to combine effective cytoreductive surgery with local control of the remaining disease and systemic treatment to prevent metastases.
Various techniques have been described for the management of PCA in dogs. However, information about different treatments is sparse due to the low incidence of this condition and publications are often case reports or studies with small numbers of patients. In addition the risk of complications associated with prostatic surgery and the relatively high age of the dogs affected and the short life expectancy after treatment are all factors that contribute to owners declining treatment for their pets, further limiting the number of dogs that can be included in a clinical study. Priority was therefore set in this thesis on developing a surgical technique with a low rate of serious complications.
Because canine PCA is diagnosed late in the course of the disease, the whole prostate is affected and localised PCA is not seen in dogs. This means that cytoreductive surgery for the management of PCA involves removal of as much prostatic tissue as possible. The Neodymium:Yttrium-Aluminium Garnet (Nd:YAG) laser was chosen as a technique for prostatic dissection as it was expected that its ability to control haemorrhage during dissection would enable more precise resection of neoplastic tissue and better preservation of sensitive neurovascular structures essential for urethral function. Only one report described the use of this type of laser for prostate surgery in dogs with prostatic disease other than carcinoma (Hardie et al., 1990). The technique used by Hardie et al. consisted of removing portions of prostate parenchyma including the capsule on either side of the urethra. Complications were frequent (all surviving dogs were incontinent) and severe (two out of six dogs died intraoperatively). The dissection technique used in this thesis was therefore modified to preserve a larger portion of the prostatic capsule, particularly the dorsal part incorporating the sympathetic nerve fibres responsible for urethral sphincter muscle tone.
Upon interaction with tissue, laser light will transmit significant energy to the tissue causing its temperature to rise, regardless whether the laser tip is used in contact or non-contact mode.

Since the dissection technique devised for the prostate required resection of as much prostate tissue as possible while preserving sensitive nerve structures, *ex vivo* studies (**Chapter 6**) were conducted to assess the thermal conductivity of prostate tissue and to predict whether the dissection technique chosen would be likely to cause thermal damage to structures that were meant to be preserved. The study concluded that when used in contact mode and for short periods of time (less than 1 minute), the laser tip was not likely to cause thermal damage to structures any deeper than 1-2 mm. Thermal damage associated with the dissection technique was then tested *in vivo* on healthy dogs before applying it to PCA patients (**Appendix to Chapter 7**). The tissue damage typically extended 0.3 to 0.8 mm beneath the surface immediately in contact with the laser tip, thereby confirming the predictions made based on the *ex vivo* model. The technique was therefore considered safe with regards to the heat generated in the tissue at a distance of one millimetre. This allowed dissection of prostate tissue within one millimetre of the dorsal capsule and urethra.

In the two clinical studies reported in **Chapters 7 and 8**, a total of 14 patients with PCA were treated by partial subcapsular prostatectomy, using the Nd:YAG laser. The working hypothesis that the laser would enable good haemostasis during dissection through the prostatic parenchyma and would allow preservation of the dorsal prostatic capsule and full function of the urethral sphincter was confirmed. Only one dog in the first clinical study (**Chapter 7**) died of systemic complications (hypoalbuminaemia and shock) but that dog had a preoperatively diagnosed clotting disorder. No other dogs had any complication related to excessive haemorrhage during dissection of the prostate. Subjectively, haemorrhage was well controlled during dissection through the parenchyma of the prostate. In neither study did any dogs develop other complications including postoperative urinary incontinence.

The non-steroidal anti-inflammatory drug meloxicam was used as an intra- and postoperative analgesic and to control local and distant tumour progression in both clinical studies (**Chapters 7 and 8**). Meloxicam was chosen as it was the most specific COX-2 inhibitor licensed for use in animals in the Netherlands at the time the studies were conducted. Even though results of **Chapter 3** suggest there is a role to play for COX-2 inhibitors in the treatment of PCA in dogs, little can be said about its efficacy based on the studies reported in **Chapters 7 and 8**, other than that it was not able to stop cancer progression completely as either local or distant cancer progression was the cause of euthanasia in many of the patients included on the studies. Further controlled clinical studies are necessary to assess the ability of COX-2 inhibitors to slow down local tumour growth and/or prevent metastatic spread in the management of PCA in dogs.

Two different modalities for local control of remaining PCA tissue were tested. In the study reported in **Chapter 7**, Interleukin-2 (IL-2) was injected into the remaining neoplastic tissue surrounding the urethra and dorsal to the urethra. In the study reported in **Chapter 8**, the efficacy of photodynamic therapy (PDT) to control the tumour locally was examined. In **Chapter 7** it was shown that partial subcapsular prostatectomy using a Nd:YAG laser combined with local administration of IL-2 and systemic treatment with meloxicam was able to relieve clinical signs, thereby improving quality of life of the patients, and that survival times of up to 8 months could be achieved. This result is comparable to other previously published studies. All other reports included only very small numbers of patients and the study reported in **Chapter 7**, which included 8 dogs, was the one with the largest number of

patients with PCA at the time it was published. These results are also comparable to those of a study published almost at the same time, which reported the results of partial prostatectomy in 10 dogs with PCA (Vlasin et al., 2006) and a median survival time of 130 days (range 2-220). Dogs treated with a combination of surgery and intraoperative photodynamic therapy using the photosensitiser hexyl-aminolevulinic acid (hexyl-ALA) (**Chapter 8**) survived significantly shorter and the study was therefore terminated after the inclusion of 6 dogs, although the initial plan had been to include as many patients as in the study reported in **Chapter 7**. The study highlighted the many challenges of intra-operative light administration to an organ like the prostate: the difficulty of placing the light source close enough to the target organ while maintaining sterility, the problem of light penetration into the tissue, particularly into the deeper layers dorsal to the urethra, the impossibility of equal administration of light to all parts of the organ because of the illuminated surface not being flat, and the problem of absorption of light by blood remaining on the surface of the tissue, despite meticulous haemostasis. Photodynamic therapy should however not be dismissed as a potential technique for managing canine PCA, either as an adjuvant to surgery or as sole treatment, and as new technologies can be developed that improve light administration to the target organ, the problems mentioned above may be overcome.

In both clinical studies, a significant minority of the patients did not respond well to treatment and had to be euthanised less than one month after surgery. These were mostly patients with preoperative clinical signs of severe stranguria. As ultrasound, CT and MRI do not allow for a good visualisation of the urethra, contrast urethrogram is the most appropriate technique to image a narrowing of the urethra, but cannot differentiate between prostate carcinoma compressing the urethra and tumour actually penetrating through the urethral mucosa. If available, endoscopy using a long narrow flexible scope (such as a human ureteroscope), combined with biopsy may enable confirmation of tumour invasion into the urethral lumen. However, good visualisation of tissues during endoscopy ideally requires dilation with air or fluid to prevent the tissue from adhering to the lens. This is difficult to achieve in the urethra, when using a flexible endoscope.

Although the treatment modality introduced in this thesis successfully relieved clinical signs and improved quality of life of many patients, it was not effective in relieving the worst cases of stranguria. Straining to urinate could not either be relieved medically with the use of sympathicolytic drugs (such as prazosine). It was therefore concluded that stranguria was caused by in-growth of tumour into the lumen of the urethra rather than by extraluminal compression of the urethra, or urethral spasm secondary to chronic irritation of nervous endings by tumour invasion into the dorsal part of the prostatic capsule. In a couple of patients, the prostatic urethra was stented using a permanently placed nephrostomy tube with mixed success. The use of the more recently developed expandable urethral stents (Crisostomo et al., 2007; Weisse et al., 2006) could be a useful addition to the technique described in this thesis and significantly improve quality of life and prolong survival times. One patient developed urinary retention without obstruction of the urethra and clinical signs of stranguria. This could be explained by tumour-induced damage to the afferent fibres of the sacral innervations of the bladder wall dorsal to the prostate.

In the clinical studies reported in this thesis, dogs were included even if they had evidence of metastases to the sublumbar lymph nodes. Metastatic lymph nodes were not removed

surgically as they were not large enough to cause any clinical signs and as removal would have significantly prolonged the procedure and increased the risk of serious complications. The presence of metastases to the lymph nodes was related to a shorter survival time in as far as metastatic spread to the lymph node was predominantly seen in the dogs with the most serious clinical signs (stranguria in particular). One dog with metastases to the sublumbar lymph nodes without signs of stranguria survived for 6 months postoperatively.
There is currently no TNM staging system for prostate carcinoma in dogs. Based on the experiences from the clinical studies in this thesis, an adaptation of the TNM staging system for humans (see **Chapter 1**) could be proposed as follows:

	Stage	**Description**
T	T0	No tumour
	T1	Prostate carcinoma limited to the prostate
	T2	Prostate carcinoma limited to the prostate causing urethral obstruction
	T3	Prostate carcinoma extending beyond the prostate (into the bladder neck and/or ureters)
N	N0	No metastases to the sublumbar lymph nodes
	N1	Metastases to the lymph nodes
M	M0	No signs of distant metastases
	M1	Metastases to the lungs
	M2	Metastases to other locations including the lumbar spine and pelvis

Other than clinical signs of straining to urinate, no prognostic factors were identified. In particular, the size of the prostate was not related to survival time after treatment.

Concerning the pathogenesis of PCA in dogs, it can be concluded from the investigations presented in this thesis:

- That the expression of cyclooxygenase-2 is not related to the degree of inflammation in PCA tissue, as opposed to human PCA.
- That the signalling pathway leading to the induction of COX-2 expression is different in neoplastic prostate cells compared to non-neoplastic prostate cells.
- That a shorter CAG repeat in the transactivation area of the androgen receptor gene may contribute to the development and progression of PCA and other prostate diseases.
- That the predisposition of the bouvier des Flandres breed to developing PCA is not related to either a higher serum concentration of androgens, or shorter polyglutamine repeats in the AR gene.

Concerning the management of PCA in dogs it can be concluded from this thesis that:

- Partial prostatectomy using a Nd:YAG laser combined with local administration of IL-2 and systemic treatment with the non-steroidal anti-iflammatorydrug meloxicam is safe and an effective symptomatic treatment for canine PCA.
- The presence of stranguria at presentation is a negative prognostic factor for survival following the management proposed in this thesis.
- Improvements in the administration of light to the prostate during photodynamic therapy are required in order to use this treatment modality at its full potential.

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CHAPTER 10

SUMMARY / SAMENVATTING

Summary

The dog is one of the few species to develop spontaneous prostate carcinoma (PCA) and is thus an attractive model for the study of the disease in humans. Many of the features of the disease in the dog are similar to its human counterpart, however a number of aspects of the pathogenesis, diagnosis and management of the disease are different between dogs and people. In both dogs and humans, PCA occurs in older individuals and metastasis through the same routes: lymph nodes, lungs and skeleton. Other similarities include the presence of prostatic intraepithelial neoplasia (PIN), thought to be a pre-neoplastic lesion. But whereas PCA is very common in men it is a rare condition in dogs. Diagnosis can be obtained early in humans by measuring prostate-specific antigen (PSA) in the blood. There is unfortunately no such marker of the disease in dogs. Treatment is also different in men with PCA compared to dogs: androgen ablation is very effective in inhibiting tumour growth in the early phases of the disease in humans, whereas it has no effect in dogs. In fact, dogs are more susceptible to develop PCA if they are castrated.

Chapter 1 of this thesis contains a general introduction presenting an overview of the literature in the context of a comparison between human and canine PCA on all aspects of the disease: Epidemiology, pathology, pathogenesis, diagnosis and treatment.

Chapter 2 presents the two aims of this thesis. The first aim was to gain insight into the pathogenesis of PCA in dogs by investigating whether known features of the pathogenesis of human PCA are also found in the dog. In particular the role of COX-2, the role of the length of CAG repeats in the androgen receptor gene and the role of circulating plasma androgens were studied. The second aim was to develop therapeutic tools for canine PCA. A surgical technique of partial subcapsular prostatectomy using an Nd:YAG laser was developed and evaluated in healthy dogs as well as dogs with PCA. Combined with surgery, two methods of local control of residual tumour tissue were studied: local administration of IL-2, and photodynamic therapy (PDT).

The results presented in **Chapter 3** show that contrary to human PCA, the expression of COX-2 in canine PCA is not associated with areas of inflammation. In fact, in dogs the expression of COX-2 was lower when the degree of inflammation was higher. These findings were confirmed in studies of the expression of COX-2 in canine PCA cells in culture, where cytokines and growth factors reduced the expression of COX-2 when they were added to the culture medium. The role of various signaling pathways was studied by adding inhibitors of these pathways into the culture medium. It was found that there are differences between non-neoplastic and neoplastic cells concerning the pathways regulating the expression of COX-2. However, these findings are still insufficient to fully understand to role of COX-2 in the oncogenesis of canine PCA.

In **Chapter 4**, the length of the CAG repeats in the androgen receptor (AR) gene was studied in dogs with and dogs without PCA. Similar to humans, it was found that the length of the

first CAG repeat (CAG-I) was significantly shorter in dogs with PCA compared to healthy dogs. However, in contrast to humans, the variation in length found in dogs was much smaller than in humans. The mechanisms through which the length of the CAG repeats could play a role in the development of PCA are still poorly understood.
Previous epidemiological studies have found the Bouvier des Flandres more at risk of developing PCA than other dog breeds. Similar differences in the prevalence of PCA between ethnic groups are seen in humans. These have been explained by differences in the length of CAG repeats in the AR gene and by differences in the concentration of serum androgens. The results presented in **Chapters 4** and **5** show that differences in the length of CAG repeats and in the plasma concentration of androgens cannot explain the predisposition of the Bouvier des Flandres for PCA. The results of **Chapter 5** demonstrate, however, that longer CAG-I repeats and shorter CAG-III repeats correlated with higher concentrations of testosterone and dihydro-testosterone. As dogs with PCA tend to have shorter lengths of CAG-I, this indicates a possible protective effect of androgens in the development of PCA.

A surgical technique involving a partial subcapsular prostatectomy using an Nd:YAG laser was developed in order to perform a precise dissection of prostate tissue as close as possible to sensitive structures such as the urethra and the dorsal part of the prostatic capsule. In order to determine the amount of thermal damage caused to prostate tissue during laser dissection and evaluate the minimal distance required to avoid damage to sensitive structures, *ex vivo* studies were performed and the results presented in **Chapter 6**. When the laser was used for periods of less than one minute, it was found that thermal lesions did not exceed 1-2 mm of tissue.

The surgical technique was then tested in healthy dogs (**Chapter 7** and **Appendix to Chapter 7**), showing that dissection up to 1 mm from the urethra and dorsal capsule was safe and did not cause any thermal damage to these structures. **Chapter 7** also reports the results of the management of dogs with PCA using this surgical technique, combined with local administration of interleukin-2 and systemic treatment with meloxicam. The laser enabled good control of haemorrhage during dissection of prostatic tissue. Median survival time was 103 days (range 5-239 days). The presence of severe stranguria preoperatively was a negative prognostic sign as the urethral obstruction was not alleviated with the treatment given and these dogs were euthanized less than 2 weeks postoperatively. In dogs without urethral obstruction, the treatment was successful in relieving the clinical signs, thereby improving the quality of life, and survival times of up to 8 months were obtained.

In another clinical study reported in **Chapter 8**, the same surgical technique and systemic treatment were used, but this time combined with intraoperative photodynamic therapy (PDT) using the photosensitiser hexyl-ALA for the local control of residual tumour tissue. Median survival time was 41 days (range 10-68 days). PDT was not successful in controlling the tumour as local progression and/or the occurrence of distant metastases meant that the patients were euthanized within 2 months after the operation. The shape of the prostate, tissue thickness and the presence of blood were all factors impairing deep and even penetration of

light into the residual neoplastic tissue to be treated, explaining the disappointing response to PDT.

These results are summarised and discussed in **Chapter 9**. The first aim of the thesis was to investigate aspects of the pathogenesis of PCA in dogs. This thesis presents some similarities and some differences between dogs and humans in the pathogenesis of PCA. The expression of COX-2 is not related to inflammation or to the degree of differentiation in canine PCA as it is in humans. However, similar to human PCA, androgen ablation is associated with increased inflammation in the neoplastic tissue. Like humans, dogs with PCA have a shorter CAG-I repeat in the AR gene, but variations in length are much smaller than in humans. Differences in CAG repeat length and plasma androgens that may explain ethnic differences in the prevalence of PCA in humans cannot explain the predisposition of the Bouvier des Flandres to develop PCA. There is growing evidence of a potential protective effect of androgens in the development of PCA in dogs.
The second aim of this thesis was to develop therapeutic tools for the management of PCA in dogs. Treatment of canine PCA remains palliative as it is effective in relieving clinical signs but cannot offer a cure. This thesis introduces an effective surgical technique for short-term management of PCA in dogs, provided there is no urethral obstruction. Photodynamic therapy is a promising treatment modality for cancer in general and prostate carcinoma in particular, however technical improvements to allow more efficient and even administration of light to prostatic tissue are necessary to make it useful in the management of clinical cases. Additional research needs to be performed to develop new techniques to remove canine PCA without recurrence or detrimental side effects. Total prostatectomy techniques, while preserving continence in the dog, partial prostatectomy with effective adjunctive radio-, chemo- or immunotherapy, or local minimally invasive interventional techniques can be mentioned as examples.

Samenvatting

De hond is één van de weinige diersoorten waarbij spontaan prostaat carcinoom (PCA) voorkomt en is daardoor dus een aantrekkelijk model voor onderzoek naar deze aandoening bij de mens. Veel van de kenmerken van deze ziekte bij de hond zijn gelijk aan de humane equivalent. Echter meerdere aspecten van de pathogenese, diagnose en behandeling van prostaatcarcinoom verschillen tussen de hond en de mens . In zowel de hond als mens, komt prostaatcarcinoom voornamelijk voor in oudere individuen en metastaseert de tumor via de zelfde routes: lymfeknopen, longen en skelet. Een andere overeenkomst is de aanwezigheid van prostaat intraepitheliale neoplasie (PIN), verondersteld wordt dat dit een pre-neoplastisch letsel is. Bij de mens komt prostaatcarcinoom regelmatig voor, terwijl het bij de hond een vrij zeldzame aandoening is. Humaan kan de diagnose vroeg gesteld worden door het meten van prostaat-specifiek antigeen (PSA) in het bloed. Helaas bestaat er voor de hond geen vergelijkbare marker. Behandeling van PCA bij de mens verschilt van die bij de hond: humaan is androgenen ablatie effectief in het remmen van de tumorgroei in de vroege fase, terwijl dat in de hond geen effect heeft. Sterker nog, honden hebben een verhoogde kans op PCA indien ze gecastreerd zijn.

Hoofdstuk 1 van dit proefschrift bevat een algemene introductie met een overzicht aan literatuur betreffende het verschil tussen humane PCA en PCA bij de hond met betrekking tot de epidemiologie, pathologie, pathogenese, diagnose en behandeling.

Hoofdstuk 2 bevat de twee doelstellingen van dit proefschrift. De eerste doelstelling was om inzicht te verkrijgen in de pathogenese van PCA bij de hond en te onderzoeken of bekende factoren in de humane pathogenese ook een rol zouden kunnen spelen bij de hond. Vooral de rol van COX-2, de rol van de lengte van het CAG-repeats in het androgeenreceptor gen en de rol van het circulerende plasma androgeen werden bestudeerd. De tweede doelstelling was het ontwikkelen van therapeutische mogelijkheden voor PCA bij de hond. Een chirurgische techniek voor partiële subcapsulaire prostatectomie middels een Nd:YAG laser was ontwikkeld en beoordeeld in zowel gezonde honden als in honden met PCA. Gecombineerd met chirurgie, twee methoden van locale behandeling van rest tumorweefsel werd bestudeerd: lokale toediening van IL-2, en fotodynamische therapie (PDT).

De resultaten getoond in **hoofdstuk 3** laten zien dat in tegenstelling tot bij de mens, de expressie van COX-2 bij honden met PCA geen verband heeft met ontstekingsgebieden. Bij honden is de expressie van COX-2 zelfs lager naarmate er meer ontsteking aanwezig is. Deze resultaten zijn bevestigd in studies over expressie van COX-2 in PCA celculturen van de hond, waarbij cytokines en groeifactoren de expressie van COX-2 verminderden, wanneer zij bij het cultuur medium werden toegevoegd. De functie van verschillende signaalcascades werd bestudeerd door remmers bij het cultuurmedium toe te voegen. Uit dit onderzoek bleek, dat er verschil is tussen neoplastische en non-neoplastische cellen betreffende de regulerende cascades voor de expressie van COX-2. Echter, deze resultaten zijn nog onvoldoende om de volledige functie van COX-2 in de oncogenese van PCA bij de hond volledig te begrijpen.

In **hoofdstuk 4** werd de lengte van CAG-repeats in het androgeen receptor (AR) gen bestudeerd in honden met en in honden zonder PCA. Vergelijkbaar zoals bij de mens, bleek dat de lengte van het eerste CAG-repeat (CAG-I) significant korter was in honden met PCA vergeleken met gezonde honden. Echter in tegenstelling tot bij de mens, was de variatie in lengte gevonden in de hond een stuk kleiner dan bij de mens. Het mechanisme waarmee de lengte van CAG-repeats een rol zou kunnen spelen bij de ontwikkeling van PCA is nog niet bekend.
Eerdere epidemiologische studies toonden aan dat de Bouvier des Flandres een hogere kans heeft op het ontwikkelen van PCA vergeleken met andere rassen. Overeenkomstige verschillen in het voorkomen van PCA tussen etnische groepen is gezien bij de mens. Deze verschillen worden verklaard aan de hand van verschillende lengtes van CAG-repeats in het AR gen en door verschillen in de concentratie van het serum androgeen. De resultaten van **hoofdstuk 4 en 5** tonen dat verschillen in CAG-repeats lengtes en in de concentratie van plasma androgeen, de predispositie van de Bouvier des Flandres niet kunnen verklaren. Echter de resultaten in **hoofdstuk 5** laten zien dat langere CAG-I-repeats en kortere CAG-III repeats overeenkomen met een hogere testosteron concentratie en dihydro-testosterone. Aangezien honden met PCA een kortere CAG-I neigen te hebben, impliceert dit mogelijk een beschermend effect van androgenen in het ontwikkelen van PCA.

Een chirurgische techniek betreffende een partiële subcapsulaire prostatectomie met een Nd:YAG laser werd ontwikkeld met als doel een precieze dissectie van prostaatweefsel uit te voeren, zo dicht mogelijk bij gevoelige structuren zoals de urethra en de dorsale zijde van het prostaatkapsel. Om de mate van thermische schade aan prostaatweefsel tijdens laser dissectie te beoordelen en om de minimale afstand te bepalen die nodig is om schade aan gevoelige structuren te voorkomen, werden er *ex vivo* studies uitgevoerd (**hoofdstuk 6)**. Indien de laser korter dan 1 minuut werd gebruikt, was thermische schade aan weefsel minder dan 1-2 mm.

Vervolgens werd de chirurgische techniek toegepast in gezonde honden (**hoofdstuk 7 en bijlage van hoofdstuk 7**), waarbij dissectie tot 1 mm van de urethra en het dorsale kapsel veilig was en geen thermische schade veroorzaakte aan deze structuren. In **hoofdstuk 7** worden ook de resultaten getoond van behandeling van honden met PCA middels deze chirurgische techniek, gecombineerd met een lokale toediening van interleukine-2 en een systemische behandeling met meloxicam. De laser zorgde voor een goede hemostasis tijdens dissectie van prostaatweefsel. Gemiddelde overlevingstijd was 103 dagen (bereik 5-239 dagen). De aanwezigheid van ernstige strangurie postoperatief was een negatief prognostisch teken aangezien de urethraobstructie met deze behandeling niet was opgelost en deze honden in minder dan 2 weken postoperatief werden geëuthanaseerd. In honden zonder urethraobstructie, was de behandeling succesvol in het verlichten van de klinische symptomen, verbeterde hiermee de kwaliteit van leven en overlevingstijden tot 8 maanden werden verkregen.

In een andere klinische studie weergegeven in **hoofdstuk 8,** werd dezelfde chirurgische techniek gebruikt, echter deze keer gecombineerd met intraoperatieve fotodynamische

therapie (PDT), gebruik makend van photosensitiser hexyl-ALA voor de locale behandeling van tumor restweefsel. Gemiddelde overlevingstijd was 41 dagen (bereik 10-68 dagen). PDT was niet succesvol in de behandeling van de tumor, aangezien locale progressie en/of het optreden van afstandsmetastasen betekende dat de patiënten binnen 2 maanden na de operatie geëuthanaseerd werden. De vorm van de prostaat, weefseldikte en de aanwezigheid van bloed waren allen factoren die penetratie van licht in het te behandelen tumor restweefsel bemoeilijkten, dit verklaart de teleurstellende reactie op PDT (**hoofdstuk 9)**.

De eerste doelstelling van dit proefschrift was om de aspecten van de pathogenese van PCA bij de hond te onderzoeken. Dit proefschrift laat in de pathogenese van PCA sommige overeenkomsten en sommige verschillen tussen de hond en de mens zien. In honden met PCA is de expressie van COX-2 niet gerelateerd aan ontsteking of aan de mate van differentiatie zoals bij de mens. Echter, overeenkomstig met de humane PCA, is androgenen ablatie geassocieerd met een verhoogde mate van ontsteking in tumorweefsel. Honden met PCA hebben net zoals humane PCA een korter CAG-I -repeat in het AR gen, maar variaties in lengte zijn veel kleiner dan humaan. Verschillen in de lengte van CAG-repeat en in plasma androgenen kunnen de etnische verschillen verklaren in het optreden van humane PCA, maar kunnen de predispositie van de bouvier des Flandres niet verklaren. Er is groeiend bewijs voor een mogelijk beschermend effect van androgenen in de ontwikkeling van PCA in de hond. De tweede doelstelling van dit proefschrift was het ontwikkelen van therapeutische hulpmiddelen voor de behandeling van PCA bij de hond. Behandeling van PCA blijft palliatief, aangezien het effectief is in het verlichten van klinische klachten, maar geen genezing kan bieden. Dit proefschrift introduceert een effectieve chirurgische techniek voor een korter termijn behandeling van PCA bij de hond, vooropgesteld dat er geen sprake is van een urethraobstructie. Fotodynamische therapie is een veelbelovende behandelingsmogelijkheid voor kanker in het algemeen en voor prostaatkanker in het bijzonder, echter technische verbeteringen om een gelijkmatige en efficiënte toediening van het licht in het prostaatweefsel te bewerkstelling, zijn nodig om het nuttig te maken in de behandeling van klinische casuïstieken. Aanvullend onderzoek zal uitgevoerd moeten worden om nieuwe technieken te ontwikkelen die PCA bij de hond zonder recidief of schadelijke bijwerkingen verwijderen. Totale prostatectomie technieken met behoud van continentie bij de hond, partiële prostatectomie met effectieve aanvullende radio-, chemo- of immunotherapie, of lokale minimale invasieve interventie technieken kunnen als voorbeelden genoemd worden.

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Right! Here we are at last. This is the most important section, isn't it? At least, it is the one that will be read by most people. Usually before they read anything else, I suspect. Yet everybody seems to hate the endless "thank you" speeches at the Oscars. Life is full of irony.

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Curriculum vitae

Henry L'Eplattenier was born on May 2nd, 1965 in the lakeside town of Neuchâtel, in the French-speaking part of Switzerland, where he followed a classical secondary education with Latin and Greek. He then studied Veterinary Medicine at Bern University, graduating in 1988. Immediately after University, he started working for the pharmaceutical company Ciba-Geigy in Basle (now a part of Novartis) as a doctoral student in the Toxicology Department, where he completed a Swiss doctorate on nephrotoxicity testing of drugs using cell cultures. His thesis obtained the prize for best doctorate of the faculty of Veterinary Medicine at Berne University in 1991. He pursued this project further as a post-doctoral fellow for another two years, before remembering that he really wanted to be a practising vet, treat cats and dogs and, if possible, become a surgeon. The opportunity did not arise straight away, however, and Henry had to find another job in the mean time. He was a large animal vet for one year in the beautiful and rural Jura mountains just south of Basle, where his patients were mainly cows, with a few pigs and horses, the odd sheep and every day a couple of cats to be spayed. In 1994, he was offered the possibility to join the Small Animal Hospital at Zurich University. He spent four years there, completing an internship then a residency in Small Animal Surgery under the supervision of Prof Pierre Montavon. In 1998, he passed the board exam to become a diplomate of the European College of Veterinary Surgeons (ECVS). That same year, he emigrated from Switzerland and moved to Oslo, Norway to live with his partner. While learning Norwegian, he returned to pharmaceutical industry (Nycomed Imaging), where he was a registration officer for x-ray and MRI contrast products. In 1999, he joined the Norwegian Veterinary College as an assistant professor in Small Animal Surgery. Henry was not thinking of moving, but in 2001, the opportunity to join the Soft Tissue Surgery team in the Department of Clinical Sciences of Companion Animals at Utrecht University proved too big a temptation, so he decided to change country again. He spent the next 5 years until 2006 in Utrecht in The Netherlands, where he was mainly responsible for the urology consultations and operations. This thesis is the result of his research during those years. Since 2006, Henry has been working as a consultant surgeon and head of Surgery at VRCC Veterinary Referrals in Laindon, Essex, UK, and in 2008 he was also made a director of the referral centre.

List of abbreviations

A	Androstenedione
ALA	Aminolevulinic acid
AR	Androgen receptor
CAGr	CAG repeat
COX-1, COX-2	Cyclooxygenase-1 and -2
CPSE	Canine prostate-specific esterase
DHT	Dihydrotestosterone
EGF	Epithelial growth factor
ERK/MAPK	Extracellular signal related kinase
HG-PIN	High grade prostatic intraepithelial neoplasia
IL-2, IL-6	Interleukin-2 and -6
Nd:YAG	Neodymium:Yttrium Aluminium Garnet
PC, PCA	Prostate carcinoma
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PI3K	Phosphatidyl-inositol-3 kinase
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PSA	Prostate serum antigen
SD, sd	Standard deviation
SHBG	Sex hormone-binding globulin
T	Testosterone
TNFα	Tumour necrosis factor alpha
TRUS	Transrectal ultrasound exam