Genetic analysis of prostatic diseases in *Canis familiaris*

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Abstract

The prostate gland is part of the male reproductive system and can be affected by several conditions including benign prostatic hyperplasia (BPH), prostate cancer (PCa), prostatitis and cysts. The domestic dog (*Canis familiaris*) is considered a good animal model for studying prostatic diseases because, in addition to the morphological and functional similarities between the canine and human prostate glands, these animals spontaneously develop BPH and PCa. In this context, the study of the genetic basis of the canine prostatic diseases might contribute to a better understanding of these diseases and to the development of new diagnostic tools.

The present work included a population of 20 adult male dogs presenting different prostatic conditions. The clinical diagnosis was made by rectal palpation and ultrasonography and confirmed by cytological examination of prostatic fluid. Different biological samples (blood, urine and prostatic fluid) were collected and processed using optimized protocols.

Odelis®CPSE assay was performed in all plasma samples in order to test its validity in the prostatic diseases detection. The results revealed that CPSE plasma concentrations are higher in pathological conditions than in normal control dogs. Nevertheless, it was not possible to differentiate BPH from the other prostatic diseases.

Following DNA and RNA extraction procedures, four different genetic approaches were implemented. Due to its role as a promising diagnostic tool, the *CPSE* gene expression was analyzed in dogs with different prostatic conditions but it was not possible to associate the expression profile of this gene with a specific condition. In the second approach, canine *PCA3* gene was characterized due to its central role in human PCa detection. The assembled canine *PCA3* gene was located in dog chromosome 1 and presented 79% of identity with human *PCA3* sequence. *PCA3* was not expressed in this population, indicating that this gene expression might be associated with neoplasic prostatic condition. In the third approach, two regions of the *IL-6* gene were analyzed, allowing the identification of two gene variants: one located in the coding region of exon 5 (I/5_g105G>A) and another in the untranslated region of exon 5 (I/5_g440G>A). I/5_g105G>A and I/5_g440G>A gene variants were not correlated with susceptibility to develop a specific prostatic disease. However, the I/5_g105G>A polymorphism affected the encoded amino acid and may have damage effects for the IL-6 protein; the I/5_g440G>A polymorphism may influence the susceptibility to develop prostatic diseases by affecting the regulatory elements of *IL-6* gene. In order to systematically summarize the existing data concerning the *IL-6* influence in human PCa susceptibility, the fourth approach included a systematic review and a meta-analysis based upon case-control studies. Overall, there was no significant association between *IL-6* -174G>C polymorphism and PCa increased risk. Nevertheless, concerning the *IL-6* -636G>C polymorphism, the CC and GC genotypes were statistically significantly associated with PCa decreased risk in an Asian population.

It is essential to complement this research with new approaches, in order to improve Odelis®CPSE applicability; accomplish more information about candidate genes and clarify the role of gene variants in prostatic diseases susceptibility.

Resumo

A glândula prostática faz parte do sistema reprodutor masculino e pode ser afectada por várias condições incluindo a hiperplasia prostática benigna (HPB), o cancro da próstata (CaP), a prostatite e quistos. O cão (*Canis familiaris*) é considerado um bom modelo animal para o estudo destas doenças porque, para além das semelhanças morfológicas e funcionais entre as glândulas prostáticas do homem e do cão, estes animais desenvolvem HPB e CaP de forma espontânea. Neste contexto, o estudo da base genética das doenças prostáticas caninas poderá contribuir para o melhor conhecimentos destas patologias e para o desenvolvimento de novas ferramentas de diagnóstico.

O presente trabalho incluiu uma população de 20 cães machos adultos apresentando diferentes condições prostáticas. O diagnóstico clínico foi realizado através do exame do toque rectal e ultrassonografia e confirmado pela análise citológica do fluido prostático. Foram recolhidas e processadas diferentes amostras biológicas (sangue, urina e fluido da próstata) utilizando protocolos optimizados.

O teste *Odelis®CPSE* foi analisado em todas as amostras de plasma de forma a testar a sua validade na detecção de doenças prostáticas. Os resultados revelaram que as concentrações plasmáticas de *CPSE* são mais elevadas em condições patológicas do que em condições normais. No entanto, não foi possível diferenciar a HPB de outras doenças prostáticas.

Após os processos de extração de ADN e ARN, foram implementadas quatro abordagens genéticas diferentes. Devido ao seu papel promissor como ferramenta de diagnóstico, a expressão de gene *CPSE* foi analisada em cães com diferentes condições prostáticas mas não foi possível associar um perfil de expressão deste gene com uma condição prostática específica. Na segunda abordagem, o gene *PCA3* canino foi caracterizado devido ao seu papel central na detecção do CaP no homem. O gene *PCA3* canino, localizado no cromossoma 1 do cão, apresentou 79% de identidade com a sequência humana do gene *PCA3*. O *PCA3* não foi expresso nesta população, indicando que a sua expressão génica poderá estar associada apenas com condições neoplásicas. Na terceira abordagem, foram analisadas duas regiões do gene *IL-6*, permitindo a identificação de duas variações genéticas: uma localizada na região codificante do exão 5 (I5_g105G>A) e outra na região não codificante (I5_g440G>A). As variações I5g_105G>A e I5_g440G>A não foram correlacionadas com a susceptibilidade para desenvolver uma determinada doença prostática. No entanto, o polimorfismo I5g_105G>A altera o aminoácido codificado e poderá ter efeitos prejudiciais para a proteína IL-6; o polimorfismo I5_g440G>A pode influenciar a susceptibilidade para desenvolver doenças prostáticas, através da regulação dos elementos reguladores do gene *IL-6*. De forma a sistematizar a informação existente sobre a influência do gene *IL-6* e a susceptibilidade para o homem desenvolver CaP, a quarta abordagem incluiu uma revisão sistemática e uma meta-análise com base em estudos de caso-controlo. Em

geral, não houve nenhuma associação significativa entre o polimorfismo *IL-6* -174G>C e o risco para desenvolver CaP. No entanto, tendo em conta o polimorfismo *IL-6* -636G>C, os genótipos CC e GC foram significativamente associados com o risco diminuído para desenvolver CaP numa população asiática.

É essencial complementar este trabalho com novas abordagens, de forma a melhorar a aplicabilidade do teste *Odelis*®*CPSE*; obter mais informações sobre genes candidatos e clarificar o papel das variantes genéticas na susceptibilidade para desenvolver doenças da próstata.

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Abbreviations and Symbols

A – Adenine
aCGH – Array comparative genomic hybridization
Arg – Arginine
BAC – Bacterial artificial chromosome
BLAST – Basic local alignment search tool
BMCC1 – BNIP2 motif-containing molecule at the C-terminal region 1
BPH – Benign prostatic hyperplasia
C – Cytosine
cDNA – Single-stranded complementary deoxyribonucleic acid
CIs – Confidence intervals
CPSE – Canine prostatic specific esterase
D – Glutamine
DHT – Dihydrotestosterone
DNA – Deoxyribonucleic acid
DNase – Deoxyribonuclease
dNTP'S – Deoxyribonucleotides
DRE – Digital rectal examination
EDTA – Ethylenediaminetetraacetic acid
ELISA– Enzyme-linked immunosorbent assay
FDA – Food and drug administration
fPSA – ratio of free to total PSA
G – Guanine
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
Gln – Glutamine
GLOBOCAN – International Agency for Research on Cancer
GWAS – Genome-wide association studies
H_2O – Water
HRP – Horse radish peroxidase
HVUTAD – Veterinary Teaching Hospital of the University of Trás-os-Montes and Alto Douro
HWE – Hardy– Weinberg equilibrium
IL-6 – Interleukin-6
MAPK – Mitogen activated protein kinase
$MgCl_2$ – Magnesium chloride
mRNA – Messenger ribonucleic acid
NCBI – National Center for Biotechnology Information
OD – Optical density
ORs – Odds ratio
PAP – Prostatic acid phosphatase
PCa – Prostate cancer

PCA3 – Prostate cancer antigen 3
PCR – Polymerase chain reaction
proPSA – Precursor form of PSA
PRUNE 2 – Prune homolog 2
PSA – Prostate specific antigen
R – Arginine
RNA – Ribonucleic acid
RNase – Ribonuclease
rpm – Rotations per minute
RT-PCR – Reverse transcription polymerase chain reaction
SIFT – Sorting tolerant to intolerant
SNPs – Single nucleotide polymorphisms
spp – Species
SPSS – Statistical package for the social sciences
STAT – Signal transducer and activator of transcription
T – Timidine
Tm – Melting temperature
TMB – Tetramethyl benzidine
TRUS – Transrectal ultrasound
TUIP – Transurethral incision
TURP – Transurethral resection
U – Unit
UPVET – Veterinary Clinics of University of Porto
UTR – Untranslated region
vs – Versus

Chapter I – Introduction

Chapter I – Introduction

1.1 – Relevance of prostatic diseases

Prostatic diseases are very frequent and can have infectious, hormonal, anatomical or embryological origin. Common prostatic disorders include benign prostatic hyperplasia (BPH), prostatitis, cysts and prostate cancer (PCa) (Mayenco-Aguirre and Sánchez de-la Muela, 2007).

In men, PCa is the second most common cancer diagnosed and the sixth most common cause of cancer deaths (Baade *et al*., 2009). It is rare in younger males but its incidence increases continuously with age (LeRoy and Northrup, 2009). The International Agency for Research on Cancer (GLOBOCAN) estimates that in 2008, there were 5140 new cases of prostate cancer diagnosed in Portugal and 2021 deaths related to this disease (Ferlay *et al*., 2010).

BPH is the most common disease of canine prostate gland, affecting 95% of dogs with nine years of age (Gobello and Corrada, 2002). Due to the high frequency of diseases affecting this gland, new studies focused on the development of diagnostic methods and new therapeutic approaches are needed.

Understand the epidemiology, natural history and genetics of a disease should contribute to the appropriate diagnosis and to the development of new therapeutic strategies.

1.2 - Animal models for prostatic diseases

Animal cancer models have proven to be useful for studying oncogenesis, the molecular mechanisms of initiation, promotion, angiogenesis and the development of metastasis (LeRoy and Northrup, 2009).

To become accepted as an animal model for human medicine, diseases must be similar at clinical, pathological and molecular levels (Brinkhof *et al.*, 2006). Besides that, there are some challenges in the use of animal models including the significant anatomical, histological and metastatic potential differences that exist between species (Lamb e Zhang, 2005).

The use of animal models in the study of prostatic diseases is an essential tool for defining the molecular basis of these pathologies, as well as to the development of new diagnostic methods and therapies (Lamb e Zhang, 2005).

In the early part of the 20th century, due to the anatomical similarities between the canine and human prostate glands and the paucity of spontaneous prostatic carcinomas in other animals, dogs were the primary species examined in retrospective studies of PCa in companion animals (LeRoy and Northrup, 2009).

1.2.1 – *Canis familiaris* as an animal model

The dog (*Canis familiaris*) may represent a unique animal model for studying human prostatic diseases because it spontaneously develops BPH and PCa. It also has other relevant characteristics such as the frequent access to qualified medical care and the fact that the dog co-habits with humans, minimizing the differences caused by environmental factors (Winkler *et al*., 2005).

Canis familiaris also shows an age-dependent development of malignant prostatic lesions as well as a similar metastatic pattern (Fork *et al*., 2008). Additionally, specific types of canine malignancies are similar to their human counterparts in histopathological appearance, biological behavior, and response to therapy, giving an enormous advantage to the canine model over many existing popular animal models (Starkey *et al*., 2005).

The publication of the canine genome and the position of the dog within the mammalian evolutionary tree, make it an important guide for comparative analysis of the human genome (Lindblad-Toh *et al*., 2005).

An extensive genetic study of canine prostatic diseases should provide valuable insight into the similar disorders in men and could also contribute to develop screening and diagnosis strategies for canine prostatic disorders in veterinary clinical context.

1.3 – Anatomy, physiology and histology of healthy prostate glands

The human male reproductive system consists of gonads called testes, a series of ducts: epididymis, vas deferens, ejaculatory duct and urethra and accessory sex glands: prostate, seminal vesicles and bulbourethral glands.

The human prostate gland (Figure 1) encircles the urethra just inferior to the urinary bladder and it is located in front of the rectum (Mader, 2004). The normal donut-shaped gland is about 4 cm across, 2 cm thick and 3 cm in length; it is spongy in texture (Graff, 2001) and contains four sub-regions: peripheral zone (~70%), central zone (~20%), transition zone (~5%) and a small periurethral gland region (LeRoy and Northrup, 2009).

This gland is enclosed by a fibrous capsule and divided into two lobes formed by the urethra and the ejaculatory ducts that extend through the gland. The thin, alkaline and milky-colored prostatic secretion (90% of semen volume) is rich in zinc, citric acid, antibiotic-like molecules, fructose and enzymes that are important for sperm function (Robinson, 2002).

The prostatic growth and secretion are mediated by androgens. Testosterone is the main male sex hormone produced by the Leydig cells of the testes and it is converted by the enzyme 5-alpha-reductase to dihydrotestosterone, a much more active form that regulates the growth and secretion of the gland (Memon, 2007). Furthermore, androgens also promote differentiation

of the primitive prostatic epithelium into the three mature cell types: basal, luminal and neuroendocrine cells. Prostatic basal cells are located in the base of the epithelium and they are the stem cell component of the prostate. The luminal cells, which are the functionally active prostatic secretory cells, secrete the prostatic acid phosphatase (PAP) enzyme and prostate specific antigen (PSA) that is often measured clinically to assess prostate function. Prostatic neuroendocrine cells express markers such as chromoranin-A and serotonin (Graff, 2001 and LeRoy and Northrup, 2009).

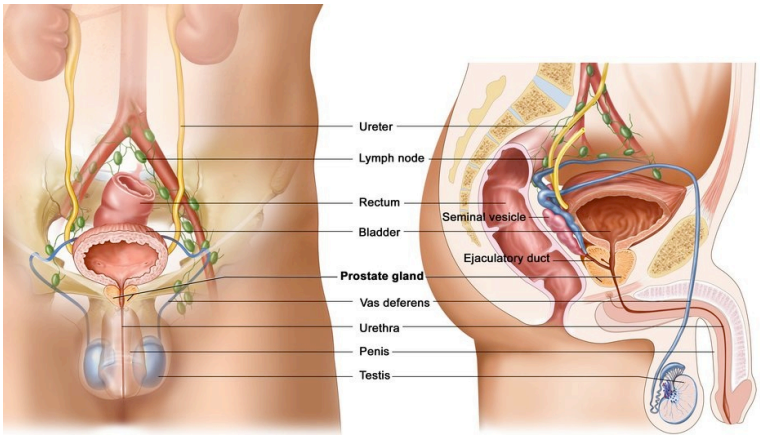


Figure 1 - Normal human prostate gland. Adapted from the National Cancer Institute – Visuals Online (http://visuals.nci.nih.gov/details.cfm?imageid=7226).

The canine prostate gland (Figure 2) shares common embryonic development and many morphological and functional similarities with the man´s gland. It is also an ovoid-shaped bilobed gland that surrounds the neck of the urinary bladder and the first part of the urethra and, as well as in man, the main purpose of this gland is to produce fluid that provides the optimum environment for sperm mobility and survival (Williams and Niles, 1999).

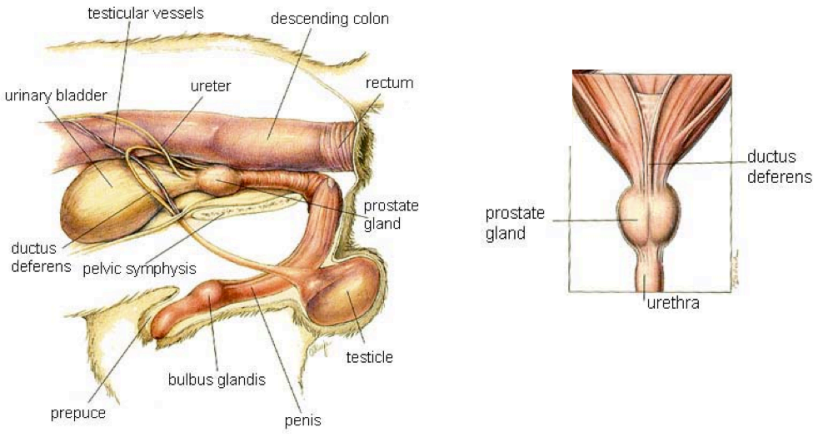


Figure 2 - Normal canine prostate gland. Adapted from the Atlas of Veterinary Clinical Anatomy.

The position and size of canine prostate gland varies with age: it is small and lies within the pelvic canal until sexual maturity, it gradually enlarges as a result of androgenic stimulation and may move cranially over the pelvic brim and into the abdomen (Williams and Niles, 1999). Additionally, its secretion is also androgen dependent (LeRoy and Northrup, 2009).

Nevertheless, the canine prostate gland has some significant differences to the human counterpart: it is the only accessory gland; it does not exhibit the zonal anatomy; its secretion does not contain fructose; the luminal cells do not secrete PSA but rather a related kallikrein family member called canine prostatic specific esterase (CPSE) and there is conflicting evidence whether or not neuroendocrine cells exist (Williams and Niles, 1999; Graff, 2001 and LeRoy and Northrup, 2009).

1.4 – Screening and diagnosis of human and canine prostatic diseases

1.4.1 – General health examination and transrectal digital palpation

Prior to the specialized urological examination, a general health evaluation including a medical history interview and general examination tests must be done.

During the medical history interview, men must mention any complaints or concerns about their health and symptoms. The physician quizzes about important behaviours (smoking, excessive alcohol use, diet and others), surgical history and medication in use. In general examination, the blood pressure, heart and respiration rates and the body temperature are checked. An abdominal examination is performed to check if the bladder is increased and the penis and testicles are also examined. Finally, the digital rectal exam (DRE) is done (Kirk, 2004).

In veterinary medicine, the general health, common habits and some detected pathological manifestations of the animal are questioned to the pet´s owner in order to perform the medical history. In general clinical examination, the hydratation score, temperature, cardiovascular and respiratory status are checked (Paclikova *et al*., 2006). Finally, the DRE procedure is performed.

DRE is considered a non-invasive screening method and the best one for physical examination of the canine prostate gland (Smith, 2008). It is a quick and simple exam in which the physician inserts a gloved and lubricated finger into the animal´s rectum and feels the prostate through the rectal tissue. This test is used to examine some aspects (volume, consistency, nodules, limits, sensivity) in order to look for abnormal conditions or signs of prostatic disease (Mayenco-Aguirre and Sánchez de-la Muela, 2007). The effectiveness of this procedure depends on the ability of physician/veterinarian finger to detect abnormalities. In case of any doubt and depending on the findings of these examinations, more specific exams should be performed (Kirk, 2004).

1.4.2 – Diagnostic imaging – radiography and ultrasonography

Radiography and ultrasonography are diagnostic imaging techniques useful for the diagnosis of men and dogs' prostate disorders.

Radiography has been used to evaluate the position, size and outer surface of prostate gland and to detect prostatic cysts and metastasis in bony structures, pelvic lymph nodes and lungs (Paclikova *et al*., 2006). In veterinary clinical context, the prostate is considered enlarged when its size is greater than 50% of the width of the pelvic inlet on a ventrodorsal radiograph (Smith, 2008).

Ultrasonography is more precise and accurate than X-rays and provides images of the gland and its surrounding tissue in real time. This technique has been successfully used to determine the width and length as well as to visualize the internal architecture and external texture of prostate gland. It is also used to detect and evaluate the size of prostatic cysts and for guidance when biopsy or aspiration procedures are performed (Smith, 2008). Normal prostate condition should have a homogeneous echodense pattern; hyperplasia, inflammation or neoplasia can result in hyperchogenic foci and cysts and abscesses can present anechoic and hypoechoic areas (Paclikova *et al*., 2006).

DRE and ultrasonography can detect prostate abnormalities. However, further procedures, such as cytological examination and biopsy, are needed to achieve the definitive diagnosis.

1.4.3 – Cytological examination

Cytological examination of urine samples has been extensively used to detect cancer and inflammatory diseases of the urinary tract. Urine specimens include many exfoliative cells from urinary system but few exfoliative cells from the prostate. So, in order to evaluate and differentiate prostatic disorders, cytological examination of prostatic fluid or semen should be done (Chen *et al*., 2006).

Cytological samples may be obtained by effective collection techniques such as ultrasound-guided fine needle aspiration and urethral catheterisation with prostatic massage. The type of sample (prostatic fluid or semen) and the collection technique used influence cytological features (Kraft *et al*., 2008).

In normal conditions, prostate fine needle aspirates contain differentiated cuboidal to low columnar prostatic epithelial cells that are organized in small or medium clusters. The nucleus is usually round to oval and the cytoplasm is finely granular, basophilic and may be vacuolated (Mayenco-Aguirre and Sánchez de-la Muela, 2007).

Ejaculate samples in normal conditions may contain a few erythrocytes, leukocytes, squamous epithelial cells, contaminant organisms (bacteria) and numerous spermatozoa. On the

other hand, prostatic fluid samples may include erythrocytes, leukocytes, spermatozoa, transitional epithelial cells and squamous epithelial cells but no bacteria or other contaminant organism (Kraft *et al*., 2008).

When BPH is present, the epithelial cells are differentiated and arranged individually or in clusters with acinar-like arrangement and indistinct cytoplasmic borders. The cytoplasm is slight granular and basophilic and the nuclei present finely reticulated or stippled chromatin. There may be an increase in cell size and the nucleus-cytoplasm ratio is increased compared with normal cells (Kraft *et al*., 2008).

Cytological evaluation of acute prostatitis reveals the presence of a large number of degenerate neutrophils sometimes with detectable intracellular bacteria. Chronic prostatitis is characterized by large number of macrophages, lymphocytes and neutrophils (Mayenco-Aguirre and Sánchez de-la Muela, 2007).

In case of prostatic cysts, the fluid contains a small number of epithelial cells, some neutrophils and cellular debris or a moderate number of slightly hyperplastic epithelial cells. Sometimes the fluid is acellular (Paclikova *et al*., 2006).

Aspirate smears of prostatic carcinomas reveal abnormal epithelial cells arranged in variably sized clusters. There is an increase in cell size and number, nuclear enlargement and the cytoplasm is deeply basophilic (Kraft *et al*., 2008).

The results of cytological examinations should be interpreted with clinical findings, blood examination tests and urinalysis.

1.4.5 – Prostatic biomarkers

A biomarker is a biological molecule that can be found in blood, body fluids or tissues and its measurement and evaluation can be indicative of normal or pathological processes (Madu and Lu, 2010).

The different prostatic conditions may present similar clinical signs which difficult the definitive diagnosis. Therefore, the validation of already existing biomarkers and the discovery/development of new markers for prostatic diseases screening and detection are emergent issues.

Human prostatic acid phosphatase (PAP), discovered in 1930 by Gutman and colleagues, was the first reported serum biomarker for PCa progression and reaction to androgen deprivation therapy of PCa that had metastasized (Madu and Lu, 2010). Some years later, with the discovery of a more sensitive and specific marker - serum prostate specific antigen (PSA), PAP measurement was gradually replaced.

PSA is a member of human kallikrein proteases and was purified from prostate extracts in 1970. Initially, PSA was used for monitoring prostate cancer patients but was subsequent implemented for screening because several studies demonstrated that its measurement provided a useful adjunct to DRE in PCa detection (Makarov *et al*., 2009). In 1994, FDA officially approved PSA test, defining 4,0 ng/mL as the upper limit of normal conditions (Madu and Lu, 2010).

Over time, some limitations in the use of PSA for PCa screening became evident and, in order to improve its specificity, several variations on the basic test have been proposed including the rate of PSA change over time (PSA velocity), the ratio of PSA concentration to prostate volume (PSA density), the time necessary for the serum PSA level double (PSA doubling time), age specific PSA intervals, precursor forms of PSA (proPSA) and the ratio of free to total PSA (fPSA) (Makarov *et al*., 2009). Nowadays, PSA test is used in conjunction with DRE and it is considered an important biomarker for detecting, staging and monitoring human PCa. Nevertheless, this test is not specific because common pathological conditions such as BPH and prostatitis can increase PSA levels from a normal low level to an elevated state in the blood, leading to a high rate of false positives and to unnecessary biopsies (Schilling *et al*., 2009).

In 1999, Bussemakers and his colleagues identified a novel biomarker - prostate cancer antigen 3 (PCA3 or DD3). They used differential display analysis to examine differences in mRNA expression levels in benign versus malignant prostate tissue and they reported that *PCA3* gene was highly overexpressed in PCa tissues (Schalken, 2009). Further studies using quantitative research tests led to the development of PCA3 urine assay.

PCA3 urinary test has a lower sensivity than blood PSA test but it has higher specificity. This assay measures PCA3 and PSA mRNA levels in urine collected following DRE and the final output is a ratio of PCA3/PSA mRNA multiplied by 1000 (Schalken, 2009). In the last recent years, several studies have supported the usefulness of this test as a specific biomarker for the diagnosis of PCa stage and grading and confirmed its utility to guide a decision for a biopsy (Schilling *et al*., 2009). The PCA3 test is already available in some commercial laboratories (Tuma, 2010); however, further investigation and validation studies are necessary to better understand the potential clinical utility of this test.

In veterinary medicine, the definition of prostate canine specific biomarkers is scarce. In 1995, Bell and colleagues measured PAP, PSA and canine prostate specific arginine esterase (CPSE) serum and seminal plasma concentrations in dogs with different prostatic conditions (Gobello and Corrada, 2002). They verified that PSA was not detected in canine serum or plasma and PAP concentrations did not differ significantly between normal dogs and those with prostatic diseases. On the other hand, they verified that CPSE serum concentrations in dogs with

BPH were significantly higher than in normal control dogs (Bell *et al*., 1995). These results suggested that CPSE, the major secretory product of canine prostatic epithelial cells, could be a promising biomarker for BPH detection. There is a commercially available assay for CPSE serum concentration measurement (Odelis® from Virbac); however there is no sufficient evidence on this potential biomarker. In this context, further studies are needed to define the exact role of CPSE and its usefulness as a diagnostic tool for non-neoplasic canine prostatic disorders.

1.4.6 – Prostatic biopsy

Prostatic biopsy is a procedure that removes a small piece of prostate tissue that will be cytological examined for the presence of abnormal cells. It provides information about the cellular nature and the stage it has reached.

In men, a biopsy may be recommended if the results of DRE and PSA test suggest PCa condition (Schilling *et al*., 2009). Despite the diagnostic imaging techniques and the clinical evaluation of PSA, prostatic biopsy is the most definitive diagnostic tool for PCa (Shariat and Roehrborn, 2008). There are different types of prostatic biopsies such as the transrectal, the transurethral and the transperineal. Transrectal ultrasound (TRUS)-guided biopsy using a 12-core scheme is the most reliable method to ensure accurate sampling of prostatic tissue in men with suspected PCa (Shariat and Roehrborn, 2008). During a biopsy, many men experience anxiety, pain and discomfort. This is an invasive procedure and can induce complications such as hematuria, rectal bleeding and urinary tract infections (Schilling *et al*., 2009). Following biopsy, cytological and histological examinations are performed and the Gleason score is established based on tumor architectural differentiation patterns that are classified from 1 to 5. This tumor scoring system is a result of the sum of the score of the two most common patterns. Higher scores are associated with more aggressive tumors (Shen and Shen, 2010).

In the last decades, the number of taken biopsies has significantly increased because of the widespread use of PSA test. Due to the significant proportion of negative biopsies, additional diagnostic tests are necessary to reduce the number of unnecessary and uncomfortable biopsy procedures (Schilling *et al*., 2009).

In veterinary clinical context, the decision to take a biopsy is done when the less invasive diagnostic methods do not render a diagnosis; when a case has been unresponsive to initial therapy or when an immediate diagnosis is required (Smith, 2008). There are two basic methods to perform a prostatic biopsy – percutaneous (perirectal or transabdominal approach) and excisional. Excisional biopsy is performed during surgery and is the most reliable method.

1.5 – The prostatic diseases in clinical context

Prostate gland can be affected by several conditions including BPH, prostatitis, cysts and PCa. The clinical signs of these different prostatic diseases are similar due to the fact that all these conditions cause some degree of prostatic enlargement or inflammation (Williams and Niles, 1999; Mayenco-Aguirre and Sánchez de-la Muela, 2007). The pathogenesis, clinical signs, diagnosis and the available therapies for each single prostatic disorder will be described in the following section.

1.5.1 - Benign Prostatic Hyperplasia (BPH)

BPH is the most common disease of human and canine prostate glands (Gobello and Corrada, 2002). It is a spontaneous disease and is part of an aging process that includes an increase in cell number (hyperplasia) and in cell size (hypertrophy) (Smith, 2008).

BPH pathogenesis is not completely understood, although it is clear that it begins with an alteration of the androgen:estrogen ratio by the testes (Gobello and Corrada, 2002; Smith, 2008). Estrogens induce dihydrotestosterone (DHT) receptors, increasing the sensitivity of the gland to DHT. An overexpression of DHT is the primary mediator of BPH because it enhances growth, stimulating the enlargement of prostate (Smith, 2008; LeRoy and Northrup, 2009).

In men, as prostate gland enlarges, it squeezes the urethra causing urine to back up into the bladder (Figure 3), then into the ureters, and finally, into the kidneys. This pathological condition occurs in both men and dogs but there are some differences between species.

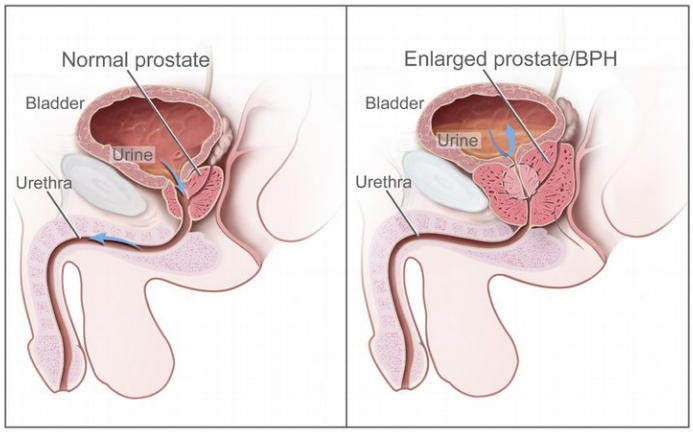


Figure 3 - Enlargement of man´s prostate gland causing urine to back up into the bladder. Adapted from the National Cancer Institute – Visuals On-line (http://visuals.nci.nih.gov/details.cfm?Imageid=71 37).

In dogs, BPH cases are not usually associated with clinical signs. However, constipation, sanguineous fluid dripping from the urethra, hematuria and hemospermia occur in some dogs.

Canine BPH affects mostly epithelial cells in a diffuse fashion (Paclikova *et al*., 2006) and there is no breed predisposition for this condition. This non-neoplastic disease is initiated between sexual maturity and 4 years of age and it appears as an intra-alveolar increase in papillary proliferations of prostatic secretory epithelium (diffuse glandular BPH). The next alteration is the increase of the stromal component that appears approximately at 6 years of age (diffuse complex BPH). Canine prostate in this state commonly contain additional pathology including cystic or atrophied glands with chronic inflammation (Memon, 2007; LeRoy and Northrup, 2009).

In humans, clinical signs include impaired bladder emptying, difficulty urinating, and hematuria (Memon, 2007). BPH is rare in men with less than 30 years old, but is present in more than half of men in their 60s and as many as 90% of those over 90 years old. This condition takes place more frequently within the stroma of the gland and it is characterized by a nodular expansion of proliferating stromal and glandular elements (Paclikova *et al*., 2006).

Diagnosis of BPH is based on the presence of typical clinical signs and exclusion of other prostatic disorders. Hematological and serum biochemical analysis are unaffected, while urine and semen analysis may be normal or contain blood without pyuria (Gobello and Corrada, 2002). Prostate size and other characteristics can be evaluated by transrectal palpation, radiography and ultrasonography. A presumptive diagnosis is made based on a thorough history, physical examination findings, prostate imaging and examination of prostate fluid but the definitive diagnosis can only be made after biopsy (Smith, 2008).

After diagnosis, the choice of the appropriate treatment is based on the severity of symptoms, the extent to which they affect lifestyle and the presence of other medical conditions. In dogs, the treatment of BPH is only recommended if related abnormal signs are present. The most effective treatment is castration but sometimes owners refuse it. There are other medical options that can include antiandrogenic, progestagens or antiestrogenic compounds that promote the decrease of prostatic size, which alleviates related signs (Gobello and Corrada, 2002; Smith, 2008). In men with only a slightly enlargement of the prostate gland, it is recommended regular check-ups and lifestyle changes. When men have moderate symptoms, it is usually used a combination of drugs including alpha-blockers, 5-alpha reductase inhibitors and anticholinergic. If the symptoms are more severe, the treatment can include minimally invasive therapies: holmium laser enucleation of the prostate, laser photovaporization, transurethral microwave thermotherapy, needle ablation, transurethral resection (TURP) or incision (TUIP) of the prostate. In cases of extreme prostate enlargement it is recommended a traditional surgery called prostatectomy (Chapple, 2004; Burnett and Wein, 2006).

1.5.2 – Prostatic Cysts

Prostatic cysts are cavitating lesions formed when canaliculi become obstructed, leading to accumulation of prostatic fluid (Smith, 2008). Based upon their location, these cysts can be classified as intra-prostatic, retention or paraprostatic cysts (Memon, 2007). Intra-prostatic cysts are usually observed as a result of BPH and are the most common cysts. Retention cysts are present within the prostatic parenchyma whereas paraprostatic cysts are outside the prostatic parenchyma and have been associated with remnants of the uterus masculinus (Paclikova *et al*., 2006; Smith, 2008).

Prostatic cysts are usually asymptomatic and typically detected during transrectal ultrasound and computed tomography scans (Lee *et al*., 2010). These techniques help to establish the size, margins, location, consistency and the number of prostatic cysts.

When present, clinical signs can include urinary obstruction, bloody urethral discharge, tenderness, lethargy, dysuria and straining during defecation (Memon, 2007; Kraft *et al*., 2008). In dogs, current treatment includes surgical procedures such as debridement and omentalization, insertion of surgical drains, cyst drainage by aspiration, cyst resection and partial prostatectomy. In addition, treatment with finasteride or castration is also recommended (Parry, 2007; Smith, 2008). For men, there are multiple treatment choices including transrectal aspiration or sclerotherapy, marsupialization with a transurethral technique and open surgery (Lee *et al*., 2010).

1.5.3 – Prostatitis

Prostatitis is an inflammatory process of the prostate gland. There are four forms of prostatitis: acute bacterial prostatitis, chronic bacterial prostatitis, chronic prostatitis/chronic pelvic pain syndrome and asymptomatic inflammatory prostatitis (Krieger *et al*., 2008).

The acute prostatitis occurs due to bacterial urinary tract infection. The most common pathogen is *Escherichia coli* but there are other responsible organisms like *Staphylococcus aureus*, Klebsiella spp., *Proteus mirabilis*, Mycoplasma spp., *Pseudomonas aeruginosa*, Enterobacter spp., Streptococcus spp. and others (Paclikova *et al*., 2006). The clinical signs in men include high fever, difficulty urinating, painful urination, urgency, pain in the pelvis and bloody urine. Dogs present signs of systemic disease (fever and depression) as well as vomiting, caudal abdominal pain, stiff or stilted gait, preputial discharge and possibly unwillingness to breed. The diagnosis is usually based on the history, physical examination, transrectal examination, urinalysis, semen and prostatic fluid analyses, bacterial cultures and hematology. DRE reveals a tender and tense prostate gland; urine culture identifies the bacteria and its sensivity to antibiotics; blood tests show a high white blood cells count and elevated levels of

PSA as a consequence of increased vascular permeability and disrupted epithelium of the gland. The treatment involves the administration of bactericidal antibiotics that can be combined with alpha-blockers and antipyretics (Ludwig, 2008; Kraft *et al*., 2008).

Chronic bacterial prostatitis is the result of recurrent urinary tract infections usually caused by the same bacterial strain. It can be asymptomatic or present symptoms including urethral discharge in dogs and pelvic pain, irritative symptoms during micturition and a variable degree of voiding and sexual dysfunction in men (Krieger *et al*., 2008; Smith, 2008; Wagenlehner *et al*., 2009). Treatment includes long duration antibiotic therapy selected based on sensivity of bacteria cultured from inflammatory exudates and on the pharmacokinetics of the antibiotic (Wagenlehner *et al*., 2009). It is also important a frequent follow-up in an attempt to prevent another recurrence. For dogs, it is also recommended castration as an adjunct to the medical treatment (Smith, 2008).

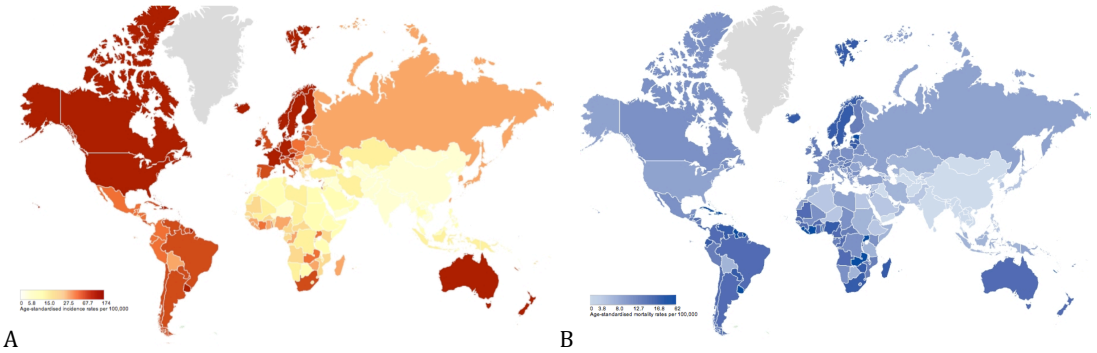
Chronic prostatitis/chronic pelvic pain syndrome is characterized by chronic pelvic pain symptoms without evidence of urinary infection. It may be inflammatory or non-inflammatory (Wagenlehner *et al*., 2009). In the inflammatory form, urine, semen and prostatic fluid contain leukocytes whereas in the non-inflammatory form there is no evidence of inflammation (Krieger *et al*., 2008). No bacterial infection can be found in the both forms. There are no diagnostic tests neither established treatments for this prostatic condition. The diagnosis is done by the exclusion of the other forms of prostatitis and treatment is based on symptomatic therapy like the management of the pain with anti-inflammatories, antianxiolytics and analgesics (Wagenlehner *et al*., 2009; Strauss and Dimitrakov, 2010).

Asymptomatic inflammatory prostatitis occurs when there is no pain or discomfort but there is inflammation in prostatic tissue and seminal fluid. This form of prostatitis is commonly found in men and dogs when looking for causes of infertility or when biopsies are done to evaluate possible PCa condition (Krieger *et al*., 2008).

1.5.4 – Prostate Cancer (PCa)

PCa is one of the most commonly diagnosed malignancies in men. It occurs when the rate of cell division surpasses cell death leading to uncontrolled growth (Madu and Lu, 2010). Its pathogenesis is complex and likely multifactorial and several genetic and environmental factors have been identified (LeRoy and Northrup, 2009).

Its incidence increases continuously with age and varies worldwide. The highest incidence rates are in North-Western Europe and Northern America and the lowest are in South-East Asia (Figure 4-A). Mortality rates are high in African descents, intermediate in Caucasian and low in Asian descents (Figure 4-B) (Schulz *et al*., 2003).



A B

Figure 4 – Estimated prostate cancer incidence (A) and mortality (B) worldwide in 2008. Adapted from *GLOBOCAN*, International Agency for Research on Cancer.

Prostatic carcinomas are usually highly dependent on androgens mediated through the androgen receptor. The androgen receptor is a member of the nuclear receptor superfamily and regulates gene expression in response to ligands in target cells (Culig *et al.*, 1994). In its inactive state, it is associated with heat-shock proteins but upon binding of androgens, it undergoes a conformational change, dissociates from the proteins, forms homodimers and translocate to the nucleus. When activated, it acts as a transcription factor and modulates the activity of various key molecules involved in proliferation, apoptosis, invasion and metastasis of PCa (Koochekpour, 2010).

Several studies have demonstrated differences in gene expression and single nucleotide polymorphisms (SNPs) in genes related to the androgen receptor, growth factors, DNA damaged, tumor suppressors, cell cycle control and inflammatory genes (Schulz *et al.*, 2003; Hughes *et al.*, 2005; Ahmed, 2010). Gene fusions and chromosomal rearrangements have been also described (Kumar-Sinha *et al.*, 2008).

Early PCa usually causes no symptoms. Advanced PCa clinical symptoms can include slow or weaken urinary stream, haematuria, loss of bladder control and when cancer metastasis have spread to bones it can result in weakness or numbness in legs (Schulz *et al.*, 2003; Hughes *et al.*, 2005).

A presumptive diagnosis is made based on two common early detection tests: DRE and PSA blood test but the definitive diagnosis can only be made after biopsy (LeRoy and Northrup, 2009). When PCa is diagnosed, conventional treatment options include watchful waiting, surgical excision of the prostate (radical prostatectomy), implantation of radioactive implants (brachytherapy) and irradiation through external beam therapy, cytotoxic chemotherapy and androgen depletion and receptor blockade therapies (Smith, 2008; LeRoy and Northrup, 2009).

Many cases of PCa grow slowly and do not become clinical relevant in a lifetime of an older man. The aggressive cancers usually metastasize to bone structures and other organs (Smith, 2008; LeRoy and Northrup, 2009). In bone metastases, there are intense epithelial-mesenchymal

interactions and the neoplasic cells stimulate maturation and activity of bone cells (osteoblasts and osteoclasts), which in turn produce growth factors that stimulate growth, and survival of neoplasic cells (Schulz *et al*., 2003).

There is an urgent need to develop new diagnostic tools to better understand the molecular basis of PCa initiation in order to distinguish between indolent and aggressive forms of this prostatic condition (Shen and Shen, 2010).

In dogs, PCa is uncommon occurring only in 5-7% of dogs with prostatic disease (Smith, 2008). This condition has an age dependency: it is rare below 10 years old but occurs in older dogs. In addition to the incidence rate, canine prostatic tumors have another important difference with human PCa, they are androgen independent and do not express androgen receptors (Smith, 2008; LeRoy and Northrup, 2009).

The most diagnosed canine prostatic tumors are adenocarcinoma and transitional cell carcinomas. Other forms include adenoma, leiomyoma, fibroma and sarcoma (Gobello and Corrada, 2002). Regardless of type, canine prostate tumors are aggressive and highly invasive.

Clinical signs can include anorexia, weight loss, haematuria, tenesmus, difficult or painful bowel movements (dyschezia), ataxia, rear limb weakness and stranguria. A more advanced stage includes metastasis to lymph nodes and bones leading to pain, myelopathy, neurological deficits in the pelvis limbs and lameness (Gobello and Corrada, 2002; Smith, 2008).

The histological examination of biopsy samples confirms the suggestive diagnosis made by DRE, radiographic and ultrasound findings.

Due to the aggressive nature of canine prostate cancer, effective treatment options are limited. Transurethral prostatectomy is indicated for early-stage lesions or tumors with no evidence of metastasis; surgical or pharmacological castration and intraoperative radiotherapy are often used to reduce prostate size (Smith, 2008; LeRoy and Northrup, 2009).

The development of biomarkers or other diagnostic methods that aid in the detection of early-stage disease and improvement of treatment options are emergent issues.

1.6 - Genetic research in prostatic diseases

In the last decades, there have been numerous advances in the basic genetic research on prostatic diseases. Defining the molecular bases of these conditions may contribute to the development of new diagnostic and prognostic methods as well as for the discovery of new targets for therapies (Lamb and Zhang, 2005).

Cancer cells typically contain multiple alterations in number and structure of genes and chromosomes, presenting defects in genome stability. Genetic changes can result in altered

patterns of gene expression and altered gene products that can lead to defects in fundamental cell regulatory mechanisms (Schulz, 2003).

Genome-wide association studies (GWAS) have identified multiple common SNPs that contribute to susceptibility to PCa and revealed that the risk variants vary in frequency across racial/ethnic populations (Waters *et al*., 2009; Chang *et al*., 2011).

Both genetic and environmental factors are probably involved in the variability of PCa aggressiveness. Using BAC-based array comparative genomic hybridization (aCGH), oligo-based aCGH and gene expression array, Rose and colleagues (2010) identified distinct regions of DNA copy number gain and loss in African-Americans versus (vs) Caucasians tumors suggesting that molecular differences may contribute to PCa health disparities.

Advanced PCa usually metastasize to bone structures and several molecular mechanisms that underlie the bone metastasis have been proposed. These molecular analyses have implicated several signaling pathways in mediating epithelial to mesenchymal interactions involved in prostate morphogenesis and PCa organogenesis (Shen and Shen, 2010).

The widespread use of PSA test has led to an overdiagnosis of patients presenting indolent tumors that may not require treatment (Albertsen *et al*., 2005). Consequently, understanding the molecular basis of cancer initiation should lead to the identification of specific biomarkers that distinguish between indolent and aggressive tumors.

Combining the existing information on human prostatic diseases with the usefulness of *Canis familiaris* as an animal model, the implementation of different genetic approaches should contribute to a deeper knowledge of these disorders.

1.6.1 – Canine Prostate Specific Esterase (CPSE) as a diagnostic tool for prostatic diseases in *Canis familiaris*

The most common prostatic disorders affecting canine prostate gland may present similar clinical signs which difficult the definitive diagnosis. Therefore, the development/ improvement of efficient diagnostic tools is urgently required.

The CPSE is the major secretory product of canine prostatic epithelial cells and it seems to be a promising biomarker for BPH detection.

The canine *CPSE* gene is comprised of five exons, spans 5510 kb and is located at chromosome 1 (GenBank: M63669.1; Gene ID: 403967, updated on 22-Dec-2011). It is a member of the kallikrein gene family and has approximately 60% of similarity with human *PSA* at the amino acid level (Lundwall and Lilja, 1987). The encoded protein has two polypeptide chains of 14 kDa and 15 kDa linked by disulphide bonds and according to its degree of glycosylation, its native form has a molecular weight between 29 and 31 kDa (Chapdelaine *et al*.,

1998; Mussel *et al*., 2010). CPSE secretion is regulated by androgens and its production can be inhibited by anti-androgen treatment or surgical castration (Gobello and Corrada, 2002).

Bell and colleagues (1995) verified that CPSE serum concentration in dogs with BPH were significantly higher than in normal dogs. Recently, Virbac Company provided an ELISA type immunoassay for CPSE serum concentration measurement called Odelis®CPSE. In a French multicentre study (2009), the CPSE serum concentration was measured in 34 dogs presenting clinical signs of BPH and in 55 normal control dogs and the results demonstrated that with a clinical threshold of 61 ng/mL (54 ng/mL – 67 ng/mL), the Odelis®CPSE assay enables the diagnosis of BPH with a sensitivity of 97.1% and a specificity of 92.7% (Lévy and Mimouni, 2009). Besides this study, there are no scientific publications associated to this potential ELISA type immunoassay. Consequently, there is a considerable interest in testing the validity and applicability of Odelis®CPSE assay in order to develop and improve non-invasive diagnostic tools for canine prostatic disorders.

1.6.2 – Characterization of *Prostate Cancer gene 3* (*PCA3*) in *Canis familiaris*

The current information regarding the genes involved in the initiation and progression of prostatic diseases in *Canis familiaris* is scarce (LeRoy and Northrup, 2009). Consequently, there is an evident lack in the definition of prostate specific biomarkers to be used in the diagnosis of the different prostatic diseases.

In human medicine, new diagnostic methods have been developed and improved using specific mRNA-based biomarkers. One such new marker is prostate cancer gene 3 (*PCA3*). PCA3 is highly over-expressed in PCa tissues compared with benign and normal tissues (Bussemakers *et al*., 1999). Consequently, a non-invasive quantitative urinary assay for PCA3 messenger RNA (mRNA) was developed and was made available for clinical use (Loeb and Partin, 2010). Several studies have demonstrated the usefulness of this assay for PCa screening in conjunction with PSA serum test and other clinical information (Kirby *et al*., 2009; Schilling *et al*., 2009).

The human *PCA3* gene is located on chromosome 9q21-22 and has no open reading frame, suggesting that this gene encodes a non-coding RNA (Day *et al*., 2011). In 1999, Bussemakers and colleagues revealed that *PCA3* gene consisted in four exons, showing alternative splicing of exon 2 and three alternative polyadenylation sites in exon 4. Twenty years later, Clarke and colleagues (2009) identified a new genomic structure for *PCA3*, involving four new transcription start sites, two new differentially spliced exons and four new polyadenylation sites (Figure 5). Additionally, they described novel *PCA3* splice variants with more specific expression in PCa tissues and metastases. Using comparative genomic analyses, they demonstrated that *PCA3* gene is located within intron 6 of the *BMCC1* gene (also called *PRUNE2*) in an anti-sense orientation

and they verified that *BMCC1* gene was also upregulated in PCa tissues, suggesting that *PCA3* transcription may influence the transcription of this gene during PCa development.

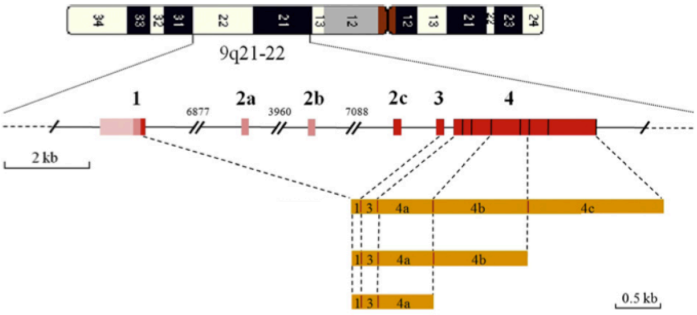


Figure 5 – The *PCA3* gene is located at chromosome 9q21–22 and was originally described by Bussemakers and colleagues, as consisting of four exons (red boxes 1, 2c, 3 and 4) and three polyadenylation sites in exon 4 (orange boxes 4a, 4b, and 4c). In 2009, Clarke and colleagues described a new genomic structure comprising two new exons (2a and 2b), four new polyadenylation sites (vertical lines in exon 4), and four new transcription start sites (exon 1, pink and light pink boxes). Adapted from Day *et al*., 2011.

Due to the relevance of PCA3 in human PCa detection, the characterization of canine *PCA3* gene and the analysis of its expression in normal and diseased canine prostate glands could be interesting to determine the usefulness of this gene for screening and diagnosis in veterinary clinical context.

1.6.3 - *Interleukin-6* (*IL-6*) gene variants and prostate cancer susceptibility

Different forms of prostate specific inflammation have been frequently associated to human PCa. Numerous oxidants produced by inflammatory cells may cause cellular or genomic damage in the prostate, enhancing cell proliferation and stimulating angiogenesis (Hughes *et al*., 2005; Shen and Shen, 2010).

SNP is a single nucleotide variant of DNA sequence and the SNP sites contain typically two alternative bases, where the less frequent is a variant or mutant or minor allele. The minor allele frequencies vary according to ethnicity, gender and disease status (Chang *et al*., 2011; Haiman *et al*., 2011; Lindstrom *et al*., 2011). For these reasons, SNPs genetic analysis is widely used for disease association studies. Recent studies shows that SNPs may explain differences in incidence, clinical manifestations, response to treatment and prognosis of many cancer types (Zabaleta *et al*., 2009 and Shao *et al*., 2011). Therefore, inflammatory gene variants might predispose individuals to PCa risk.

The human *interleukin-6* (*IL-6*) gene (Figure 6) is located on chromosome 7p21-14, consists of six exons and contains 10 transcripts (ID: ENSG00000136244; Gene ID: 3569, updated on 27-Nov-2011). It encodes a multifunctional cytokine that activates the STAT (signal transducer and activator of transcription) and MAPK (mitogen activated protein kinase) signalling pathways. The activation of these pathways leads to the transcription of target genes and to the regulation of cell growth, differentiation and survival (Hughes *et al*., 2005).

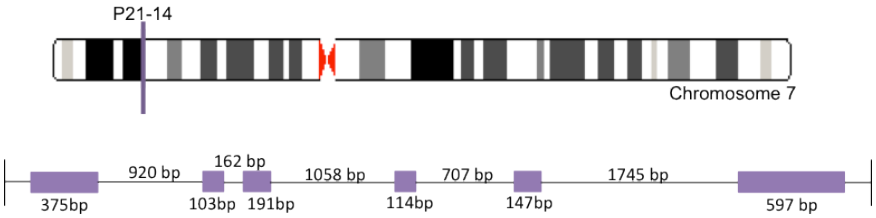


Figure 6 – Representative scheme of human *IL-6* gene. It is mapped to chromosome 7p21-14 and consisted of 6 exons (purple boxes).

IL-6 values are raised in tissues and serum from patients with PCa, the IL-6 receptor expression has been detected in PCa cell lines and several studies demonstrated its influence in the interaction between epithelial and stromal cells in PCa condition (Hughes *et al*., 2005). Accordingly, this cytokine may play a role in PCa initiation, progression and metastasis (Sun *et al*., 2004). *IL-6* gene promoter region enclose a variety of regulatory elements and two common polymorphisms, -174G>C and -636G>C, that have been implicated in PCa susceptibility by altering gene regulation and/or protein expression (Morgan *et al*., 2006; Pierce *et al.,* 2009).

Multiple studies have investigated the association between these polymorphisms and PCa risk. Whereas some studies revealed no significant increased or decreased PCa risk among men carrying *IL-6* sequence variants (Sun *et al*., 2004; Michau *et al*., 2006; Zabaleta *et al*., 2009 and others), the study of Bao and colleagues (2008) found a significant association between GG and GC genotypes of *IL-6*-636 G/C polymorphism and PCa increased risk in an Asian population. In this context, a meta-analysis of case-control studies may be an important strategy to derive a more precise estimation of the relationship between *IL-6* polymorphisms and human PCa risk.

In addition to amplify the knowledge about the genetic basis of canine prostatic diseases, the detection and characterization of *IL-6* gene variants in *Canis familiaris* may also reveal useful information about the influence of inflammation in human cancer initiation.

Chapter II – Objectives

Chapter II – Objectives

In order to contribute to a deeper knowledge of the genetic basis of the canine prostatic diseases, to the development of efficient strategies to diagnose these disorders and with the aim of reaching useful information about similar disorders in men, the main objectives of the present work were to:

- Perform a detailed literature review concerning the different human and canine prostate conditions using comparative genomic tools;
- Test the validity and applicability of Odelis®CPSE assay as a specific diagnostic tool for non neoplasic canine prostatic disorders;
- Characterize *PCA3* gene in *Canis familiaris*;
- Analyze the expression of candidate genes (*PCA3*, *CPSE*) in animals with different prostatic conditions;
- Detect and characterize *IL-6* gene variants in *Canis familiaris;*
- Perform a meta-analysis to estimate the putative association between *IL-6* promoter polymorphisms and human PCa.

Chapter III – Material and Methods

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3.1 – Biological material

In the present study, a population of 20 adult male dogs (age range, 2-12 years) with different stages of prostate development was analyzed. All animals were subjected to a general clinical examination (Annex I) after obtaining a detailed medical history.

All medical procedures were performed at the Veterinary Teaching Hospital of the University of Trás-os-Montes and Alto Douro (HVUTAD) and at Veterinary Clinics of ICBAS-University of Porto (UPVET) by a team of veterinary doctors with the consent of the animal owners (Annex II) and meeting all the standards of animal welfare. The cytological analyses were performed at the Laboratory of Histology and Anatomical Pathology of the University of Trás-os-Montes and Alto Douro by a veterinary pathologist.

The prostate size, shape, symmetry, consistency were evaluated by rectal palpation. Besides these characteristics, the internal architecture and the echogenicity of the prostate gland and testicles were evaluated by ultrasonography.

The biological samples included in this study were total blood, urine and prostatic fluid. These samples were collected and processed using optimized protocols.

Blood (1,2mL) was collected by venipuncture to two vacuum tubes with the anticoagulant EDTA. After collection, it was homogenized at room temperature for 30 minutes to prevent clotting. Plasma was separated from total blood by centrifugation at 2500 rpm for 15 minutes and stored in aliquots at -80°C. The other portion of the total blood was stored in the centrifuge tube at -20°C.

Urine was aseptically collected by ultrasound-guided cystocentesis and it was processed using two different methods. In **method I**, the urine was collected to an eppendorf tube (2mL) previously prepared with 0,75mL of RNA latter, a stabilizing solution that optimizes the performance of RNA extraction. The content was divided into three aliquots and stored at -20°C. In **method II**, the urine was collected to two eppendorf tubes (2mL) without RNA latter, which were subsequently centrifuged at 7000 rpm for 10 minutes. After removing the supernatants, the pellets were ressuspended in 500 mL of PBS and the contents of the two tubes were mixed and subsequently centrifuged at 13000 rpm for 10 minutes. The supernatant was removed, the pellet was ressuspended in 450 μL of RNA latter and stored at -20°C.

Prostatic fluid was collected by aspiration using a sterile urinary catheter (Buster®). A syringe was attached to the end of the catheter and aspirated after 1 minute of prostate transrectal massage. A portion of prostatic fluid was used to perform the cytological analysis.

The remaining content was collected to an eppendorf tube (2mL) previously prepared with 0,75 mL of RNA latter. The content was divided into three aliquots and stored at -20°C.

3.2 – Odelis® CPSE ELISA kit

In order to measure CPSE plasma concentration, an enzyme-linked immunosorbent assay (ELISA) (Odelis® CPSE kit from Virbac Animal Health) was optimized.

In the first step, the vials of calibrator and control were reconstituted and the dilutions of the stock solution were performed in order to obtain the different calibrator solutions (20, 10, 5, 2.5 and 0 ng/mL). The washing buffer solution was diluted 100 times and the samples were diluted 10 times before being assayed.

After preparation, 100 µL of each component (calibrator solution, control and sample) were placed in the corresponding wells. The microwell was covered with an adhesive film and it was incubated for 1 hour at 37°C. During this period, a first antibody (fixed to the microwell) captured CPSE proteins found in calibrators and samples.

The content of the wells was aspirated and 300 µL of washing solution were added to each well. The washing cycle was repeated four more times and after washing, a second antibody that recognizes the bounded proteins and 100 µL of ready-to-use Horse Radish Peroxidase – HRP conjugate were added to each well.

Following a second period of incubation (1 hour at 37°C), the proteins and reagents that were not specifically bound were washed (four washing cycles) and 100 µL of a substance containing the enzyme´s substrate (3,3´´,5,5´´, Tetramethyl benzidine - TMB) were added to each well.

With the addition of TMB, a colorimetric reaction begun and after 10 minutes in the dark at room temperature, 50 µL of the stop solution were added to each well.

In the final step, the optical density (OD) was read at 450 nm and CPSE plasma and calibrator's concentrations were determined by comparison with a standard curve with known concentrations. Analysis of data was performed using the statistical software SPSS (version 19.0.1). The Mann-Whitney *U* test was used to test the equality of the two distributions and the independent samples T-test, with 95% confidence interval, was used to compare the CPSE means concentrations in normal dogs and in dogs presenting signs of BPH.

3.3 – DNA extraction from blood samples

There are several methodologies for DNA isolation and purification. The extraction method is dependent on several factors: the amount of sample available, the number of samples to

extract, the availability of laboratory equipment, and in some cases, the end use of DNA (Austin *et al*., 1996).

Regardless of the protocol used, DNA extraction from any eukaryotic cell involves three essential steps: lysis of cellular and nuclear membranes, protein degradation and DNA precipitation.

Commercial kits for DNA extraction are an alternative to manual protocols. Although the DNA concentrations obtained with these protocols can be slightly lower and sometimes with a slight degradation of DNA, they have great advantages such as speed and ease of implementation and the fact that there is no need to use reagents such as phenol and chloroform (Santella, 2006).

In the present study, the DNA was extracted from the buffy coat layer of blood samples using the QuickGene DNA Whole Blood kit S (Fujifilm). In the first step, a mixture containing 30 µL of protease, 250 µL of sample and 250 µL of lysis buffer was prepared. After mixing the content by pipetting and vortexing for about 15 seconds, the tube was centrifuged briefly at maximum speed and then incubated for 5 minutes at 56°C. 250 µL of absolute ethanol were added and the tube was centrifuged briefly at maximum speed.

The following steps were performed on the Fujifilm Mini-HQ 80 equipment. The columns were placed in the washing position, 750 µL of the mixture previously obtained were transferred to the column and the liquid was pressed into the collection tube. This was followed by the addition of 750 µL of wash buffer and then, the liquid was pressed out of the column. This step was repeated two more times. The columns were placed in the elution position, 100 µL of elution buffer were added and after 2 minutes at room temperature the liquid was pressed out of the column. A second elution was obtained repeating the last procedure. The DNA obtained in the two elutions was stored in aliquots at -20°C.

3.4 – RNA extraction from prostatic fluid and urine samples

The RNA quality is of critical importance to the success of downstream applications including reverse transcription polymerase chain reaction (RT-PCR). The RNA molecule is less stable than DNA and ribonucleases are very stable, efficient, do not require cofactors for their proper functioning and they are very difficult to inactivate (Kingston, 2002). Consequently, to be successful, the RNA extraction procedure should take into account some precautions: the use of new gloves, the micropipettes' tips must have filters and the work surface should be clean and exclusive for RNA extraction.

There are several manual and commercialized protocols to isolate RNA from different types of samples. In this study, the RNA extraction was performed from prostatic fluid and urine

samples using QIAamp viral kit (Qiagen®) with some specific changes that were previously optimized.

Before starting the protocol, RNA carrier was prepared by the addition of 310 µL of Buffer AVE. Then, 16,8 µL of RNA carrier+Buffer AVE mixture were added to 1680 µL of Buffer AVL.

For each sample, three eppendorf tubes (2mL) were prepared by the addition of 560 µL of the prepared buffer AVL with RNA carrier and 140 µL of sample to each tube. After vortexing for about 15 seconds, the tubes were incubated for 10 minutes at room temperature and then, they were briefly centrifuged to remove possible drops in the cap. 560 µL of absolute ethanol were added to each tube, they were mixed on the vortex for 15 seconds and centrifuged briefly.

The next step was the addition of 630 µL of the solution obtained in the previous step to a 2 mL collection tube with a column provided by the kit. The collection tube was centrifuged at 8000 rpm for 1 minute, the column was transferred into another collection tube, the filtrate was discarded and this step was repeated until the contents of the three tubes were completely transferred to the column.

This was followed by two washing cycles where the contaminants were efficiently washed away. 500 µL of wash buffer 1 were added to the column, the tube was centrifuged at 8000 rpm for 1 minute, the filtrate was discarded and the column was transferred into another collection tube. In the second washing cycle, 500 µL of wash buffer 2 were added, the tube was centrifuged to maximum speed (14,000 rpm) for 3 minutes, the filtrate was discarded and the column was transferred into another collection tube that was centrifuged at the maximum speed for 1 minute.

In the next step, RNA was eluted in a special RNase-free buffer by the addition of 60 µL of Buffer AVE. After one minute of incubation at room temperature, the tube was centrifuged at 8000 rpm for 1 minute and the eluted RNA was stored in aliquots at -80°C.

To eliminate possible DNA contaminant molecules, subsequent treatment with the enzyme DNase I was performed using RNase-Free DNase Set kit (Qiagen®). In this treatment, 1 to 2 µg of RNA sample, 2 µL of RDD buffer, 0,5 µL of DNase I enzyme and nuclease free water to a volume of 20 µL were mixed in an eppendorf (1,5 mL). The mixture was incubated at 37°C for 30 minutes. After this, 2 µL of 25 mM EDTA were added and the mixture was incubated at 65°C for 5 minutes. The treated RNA samples were stored in aliquots at -80°C.

3.5 – *In silico* analysis and primer design

In primers selection, conditions such as primer size, nucleotide content, salt concentration and annealing temperature must be taken into account. The length of the primers should be between 18 to 24 nucleotides with a guanine (G) and cytosine (C) content between 40-60% of

the total bases. The melting temperature (Tm) of primers should be in the range of 52-60°C and the Tm of the two primers of a set should not differ more than 5°C. More than 3 G's or C's should be avoided in the last five bases at the 3' end of the primer to prevent nonspecific bindings in GC-rich regions. The primers should not form secondary structures such as self-pairing or dimers (intermolecular interactions between two primers in the same sense), cross-dimers (intermolecular interaction between sense and antisense primers) and hairpins (intramolecular interaction within the primer). To be specific, primers must only form a stable duplex with the specific site of the target DNA.

For genetic analysis, three genes (*CPSE*, *PCA3* and *IL-6*) were selected and suitable primers were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). For *CPSE* gene, two sets of primers were selected for the region that includes the exons 1-2 and exons 3-4 respectively (Annex III). Due to the absence information about dog *PCA3* gene, the Basic Local Alignment Search Tool (BLAST-NCBI) database for *Canis familiaris* was used and the human *PCA3* sequence (AF103908.1) was entered as query sequence. After the selection of an optimized program for highly similar sequences (megablast), a significant alignment was achieved and eight sets of primers, specific to the region of the alignment, were selected (Annex IV). Regarding the *IL-6* gene, two sets of primers were selected for two regions homologous to highly polymorphic regions in human *IL-6* gene (Annex V).

3.6 – Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR), a method that amplifies a desired DNA fragment in order to increase the number of DNA target molecules to detectable levels, was developed by Kary Mulis in 1983 (Pestana *et al.*, 2010).

To perform a PCR reaction, several components are needed: the DNA template, primers, deoxyribonucleotides (dNTP´s), magnesium chloride ($MgCl_2$), polymerase buffer and DNA polymerase. PCR is performed following three steps: melting, annealing and elongation. Prior to the first step, PCR reaction mixture is melted to ensure the complete denaturation of DNA template. In the first step, melting, PCR mixture is heated to 94-96°C for 0,5-2 minutes in order to denature the target DNA. In the second step, annealing, the temperature is lowered 3-5°C below the melting temperature of primers (45-65°C) for 0,5-2 minutes, allowing primers to anneal to their complementary sequences in the DNA template. In the third step, elongation, the temperature is raised to the optimum temperature for DNA polymerase activity (70-80°C) during 0,5-2 minutes. As a consequence, a new DNA strand complementary to the DNA template is synthesize by the addition of dNTP´s to the template in 5' to 3' direction. These three steps are

repeated in a series of 20-40 cycles. After the last amplification cycle, a final extension step for 5-15 minutes is usually done to fill-in the protruding ends of newly synthesized PCR products.

The amplification of candidate genes to prostatic diseases (*CPSE*, *PCA3* and *IL*-6) were optimized using the Dream Taq PCR master mix kit (Fermentas) in a *Biometra Uno II* thermocycler. PCR was carried out in a total volume of 25 µl, containing 12,5 µl of Dream Taq master mix (2x), 1 µl of $MgCl_2$ (25mM), 1 µl of each primer (100 ng/µl), 1 µl of genomic DNA (100ng) and 8,5 µl of H_2O. The amplification reaction was performed with an initial denaturation at 95°C for 5 minutes followed by 40 cycles of 95°C for 45 s, 55°C (the annealing temperature varies depending on the set of primers - Annex VI) for 45 s, 72°C for 45 s and a final extension at 72°C for 10 minutes. For CPSE_Ex1-2; CPSE_Ex3-4 and IL-6_Cf_ex2 sets of primers the number of cycles was reduced for 35 cycles and when using PCA3_Cf_2 and PCA3_Cf_5 sets of primers no $MgCl_2$ was added to the reaction mixture.

3.7 – Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of PCR that enables RNA to be used as a template. In the first step of RT-PCR, the RNA is converted into single-stranded complementary DNA (cDNA) using the enzyme reverse transcriptase. Once this preliminary step has been carried out, the resulting cDNA is amplified using a standard PCR technique.

In order to evaluate the expression of candidate genes (*PCA3*, *CPSE* and *IL-6*) in dogs with different prostatic conditions, a RT-PCR was performed using Revert Aid Reverse Transcriptase kit (Fermentas) in a *Biometra Uno II* thermocycler. The reverse transcription reaction was carried out in a total volume of 20 µl, containing 4 µl of buffer (5x), 2 µl of dNTP's (10mM), 1 µl of RNase inhibitor (20 U/µl), 1 µl of Oligo T (100 µM), 1 µl of RevertAid reverse transcriptase (200 U/µl), 3 µl of RNA and 8,0 µl of RNase free water. The cDNA synthesis was performed at 42°C for 1 hour followed by an enzyme inactivation step at 70°C for 5 minutes. All cDNA samples were stored at -20°C.

Prior to the amplification of candidate genes, a PCR amplification of a housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was optimized using the primers GAP_Cf_F and GAPDH_Ctrl_R.

PCR amplifications were carried out in a total volume of 25 µl, containing 12,5 µl of Dream Taq master mix (2x), 1 µl of $MgCl_2$ (25mM), 1 µl of each primer (100 ng/µl), 2 µl of cDNA (100ng) and 7,5 µl of H_2O. The amplification reactions were performed according to the amplification parameters referred above (point 3.6 of the present study).

3.8 – Electrophoresis

Agarose gel electrophoresis is a common technique that determines the size and purity of nucleic acids (Videira, 2001). By applying an electric field defined by the potential difference and the distance between the electrodes (V/cm), the DNA molecules migrate towards the positive electrode due to its negatively charged phosphate groups. The applied voltage, buffer composition, concentration of agarose and the DNA size and conformation influence its migration. The smaller DNA fragments move faster than the larger fragments through the agarose gel. The result of different migration in the gel will lead to different band patterns. Using this technique, PCR products can be identified by its size by comparing it with a DNA ladder that contains DNA fragments of known size. To make the amplification products visible in the agarose gel, the molecules can be stained using ethidium bromide that fluoresces under ultraviolet light when intercalated into the DNA major groove.

In order to confirm the specificity of the PCR product and determine the size of amplicons, a gel with 1,5% agarose concentration with 0,5 µg/mL of ethidium bromide was performed. For each sample, 5 µL of PCR product and 2 µL of loading dye were loaded into each well of the gel. In the last well, 2,5 µL of Gene Ruller 100 bp – DNA Ladder Plus molecular weight marker (Fermentas) was loaded. The agarose gel ran for 50 minutes at 85 V. After electrophoresis, the gel was visualized using the transilluminator Gel Doc™ Molecular Imager® (BIORAD).

3.9 – Purification of sequencing reaction products

PCR products were purified using ExoSAP-IT kit (GE Healthcare Bio-Sciences). This kit provides two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase that remove the unconsumed dNTP´s and primers from PCR mixture.

The first step consisted in adding 12 µL of PCR product to 2 µL of ExoSAP-IT. This mixture was incubated at 37°C for 15 minutes for the enzymes' activity and then, the hydrolytic enzymes were inactivated by heating to 80°C for 15 minutes. These two incubations were performed in a *Biometra Uno II* thermocycler.

3.10 – DNA Sequencing

DNA sequencing is one of the most important techniques available to the molecular biologist. This technique entails several techniques and methods that are used to determine the precise order of the nucleotides in a DNA molecule.

The automated DNA sequencing is carried out by the chain termination procedure using fluorescent labels. To perform a single sequencing reaction, a different fluorochrome is used for

each of the four dideoxynucleotides. The reaction products are loaded into a single tube of a capillary electrophoresis system and then passed in a detector that identifies the fluorescent signal of each band (Brown, 2006).

After the purification procedure, PCR products were sequenced at STABvida DNA laboratory, Caparica (http://www.stabvida.net). The chromatograms and the output sequences in FASTA format were visualized and analyzed with Chromaspro program (version 1.5; Technelysium Pty *Ltd*); the forward and reverse sequences were aligned using ClustalW2 tool (http://www.ebi .ac.uk/Tools/msa/clustalw2/) and the GeneDoc software (version 2.6.003) was used to edit the sequences. Each sequence was checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences.

3.11 – Meta-analysis and systematic review

Publication search

A literature search of two online electronic databases (PubMed and HighWire) was conducted using the search terms: "interleukin-6" or "IL-6", "polymorphism" or "prostate cancer risk" (last search was update on 31 July, 2011). In the initial screening, all the studies that reported results for the association of the *IL-6* polymorphisms and prostate cancer were considered. In addition, the references of the candidate articles were examined in an effort to identify eligible studies that may not have been identified in the initial search. Abstracts or unpublished reports were not included and only the studies published in the English language were considered.

Inclusion and exclusion criteria

For inclusion in the present meta-analysis, the identified articles had to provide all the following criteria:

(1) evaluation of *IL-6* -174G>C or -636 G>C polymorphisms with calculation of prostate cancer odds ratios;

(2) case-control design study;

(3) clear information of genotype frequencies;

(4) genotype distribution of the control population, to allow calculation of Hardy-Weinberg equilibrium (HWE).

The exclusion criteria included:

(1) no control population;

(2) no usable data reported;

(3) duplicates.

Data extraction

The qualitative information and quantitative data of all eligible studies were extracted according to the inclusion criteria and using a standardized form.

For each study, the information was collected pertaining to: the first author´s last name, year of publication, study population, study design and numbers of genotyped cases and controls. Populations were categorized according to the ethnic background. For studies that included more than one ethnic descent, data were extracted separately.

Statistical analysis

Allelic frequencies were calculated from genotype distributions and HWE analysis in control populations was calculated with Chi-square test.

In order to compare the distribution of alleles and genotypes between cases and controls for the two common *IL-6* promoter polymorphisms, a meta-analysis stratified by ethnicities was performed.

The fixed effects model was chosen to combine the results of the individual studies because the publications were clinically and methodologically similar. For each polymorphism (-174G>C and -636 G>C), the association between allele C and PCa risk, as well as homozygous comparison (CC versus (vs) GG); heterozygous comparison (GC vs GG); dominant genetic model (CC/GC vs GG) and recessive genetic model (CC vs GG/GC) were explored.

Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated and a P-value below 0,05 was considered statistically significant. Heterogeneity assumption was checked by Q-statistic test and quantified by I^2 metric value (I^2 = 0–25%, no heterogeneity; I^2 = 25–50%, moderate heterogeneity; I^2 = 50–75%, large heterogeneity; I^2 = 75–100%, extreme heterogeneity).

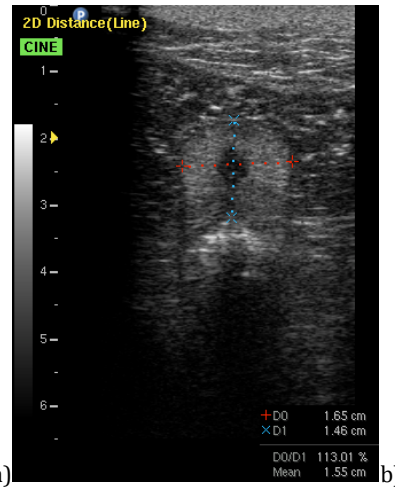
All statistical analyses were performed using the Comprehensive Meta Analysis statistical software (version 2.2.0048, November 7, 2008).

Chapter IV – Results

Chapter IV – Results

In the present study, 20 adult male dogs with different prostatic conditions were analyzed. The clinical diagnosis was made by rectal palpation and ultrasonography.

Figure 7a reveals an ultrasonography image obtained in a transversal plane, indicating the width (1,65 cm) and height (1,46 cm) of a normal prostatic condition with a homogeneous echodense pattern. Figure 7b shows two ultrasonography images obtained in longitudinal (left) and transversal (right) planes, indicating the length (6,37 cm), width (6,45 cm) and height (3,52 cm) of a BPH case and the presence of an intra-prostatic cyst (arrow).

a) 

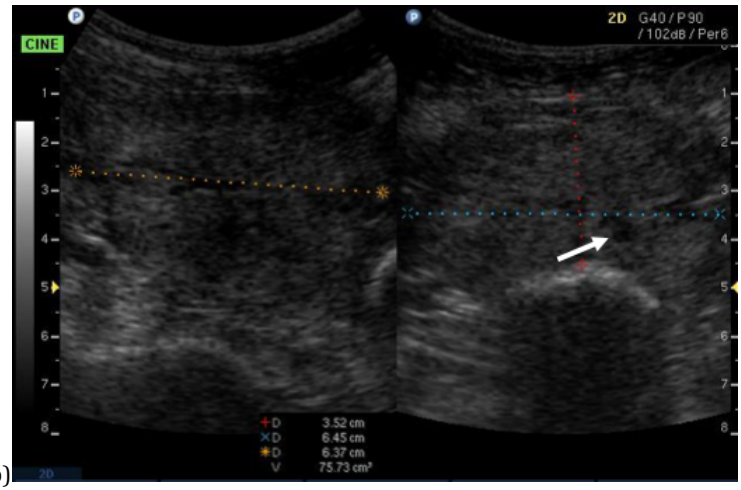
b) 

Figure 7 – Ultrasonography images of two different animals, revealing a normal prostatic condition (a) and a BPH case (b) with a small intra-prostatic cyst (arrow). The width, height and length of prostate gland were measured in transversal and longitudinal planes.

In order to confirm the clinical diagnosis, a cytological examination of prostatic fluid was performed. Figure 8 reveals a cluster of epithelial cells from a normal prostate (a) and a cluster of prostatic epithelial cells from an older dog presenting signs of BPH (b).

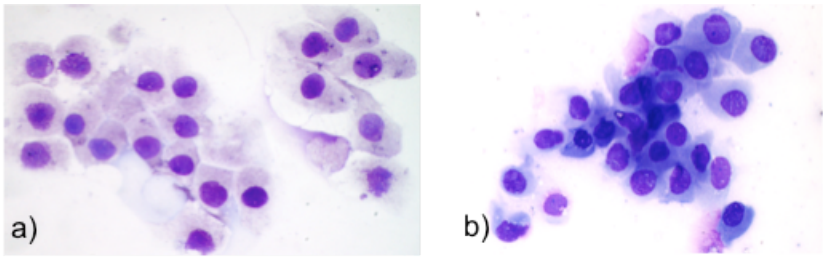


Figure 8 – Epithelial cells from a normal prostate (a) and a cluster of prostatic epithelial cells from an older dog with BPH (Wright-Giemsa stain, original magnification 40x).

The epithelial cells in the normal condition (Figure 8a) were organized in medium clusters, their nuclei were round and the cytoplasm was finely granular and vacuolated. In the BPH case,

(Figure 8b) the epithelial cells had larger nuclei with reticulated chromatin pattern and their cytoplasm was slight basophilic and had indistinct borders.

After obtaining the clinical and cytological diagnoses, an ELISA-type immunoassay (Odelis®CPSE) was performed in order to measure CPSE plasma concentration in eight dogs presenting signs of BPH, three revealing signs of BPH and prostatitis, three dogs with prostatitis, one with squamous metaplasia and in five normal control dogs (Table I).

Table I - Association between the clinical-cytological diagnoses and the Odelis®CPSE results. (The nomenclature "V" was used for the animals observed in HVUTAD and "P" for the animals observed in UPVET).

	Clinical-Cytological Diagnoses	CPSE plasma concentration (mean)	Diagnosis (Odelis®CPSE)
V1	BPH	250,00 ng/mL	BPH
V2	BPH	109,69 ng/mL	BPH
V4	BPH	176,94 ng/mL	BPH
V7	BPH	266,37 ng/mL	BPH
V10	BPH	73,86 ng/mL	BPH
V12	BPH	69,26 ng/mL	BPH
V13	BPH	158,57 ng/mL	BPH
P4	BPH	181,30 ng/mL	BPH
	Mean concentration:	**160,75 ng/mL**	
V5	BPH and prostatitis	181,30 ng/mL	BPH
V9	BPH, chronic prostatitis and bacteria	196,86 ng/mL	BPH
V19	BPH and prostatitis	241,59 ng/mL	BPH
	Mean concentration:	206,58 ng/mL	
V8	Prostatitis	143,65 ng/mL	BPH
V15	Prostatitis	36,60 ng/mL	No BPH
V17	Prostatitis	47,47 ng/mL	No BPH
	Mean concentration:	75,90 ng/mL	
V14	Squamous metaplasia	127,48 ng/mL	BPH
V6	Normal	9,16 ng/mL	No BPH
V16	Normal	36,60 ng/mL	No BPH
V18	Normal	24,16 ng/mL	No BPH
P1	Normal	47,47 ng/mL	No BPH
P6	Normal	11,27 ng/mL	No BPH
	Mean concentration:	**25,73 ng/mL**	

Stratifying by prostate conditions, the CPSE mean concentration in pathological conditions was higher than in normal state. Additionally, the mean concentration in dogs presenting signs of BPH (160,75 ng/mL) was higher than the expected values (54–67 ng/mL) indicated in Odelis®CPSE assay brochure.

Using SPSS program, the independent samples T-test was used to compare CPSE plasma concentration between normal control dogs and BPH cases. These two groups were significantly different (P-value=0,001) confirming that dogs presenting signs of BPH have higher CPSE plasma concentration than normal dogs (Figure 9).

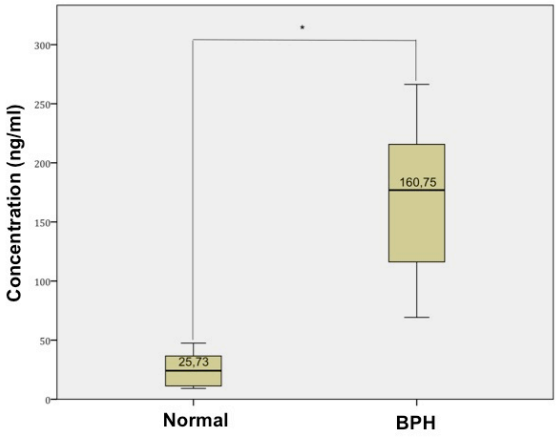


Figure 9 – The independent samples T-test was used to compare CPSE plasma concentration between normal control dogs and BPH cases. These two groups were significantly different (P-value=0,001,*P-value<0,05; statistical analyses were performed using SPSS program).

The next step consisted in the analysis of two regions of *CPSE* gene (exon1-exon2 and exon3-exon4 – Annex III) at DNA and RNA levels. After selecting specific primers, these two *CPSE* gene regions were amplified by PCR using DNA extracted from the buffy coat layer of blood samples and the PCR products were analyzed by electrophoresis on a 1,5% agarose gel. Using CPSE_Ex1-2 set of primers, the expected 416bp fragment was seen in all DNA samples (Figure 10).

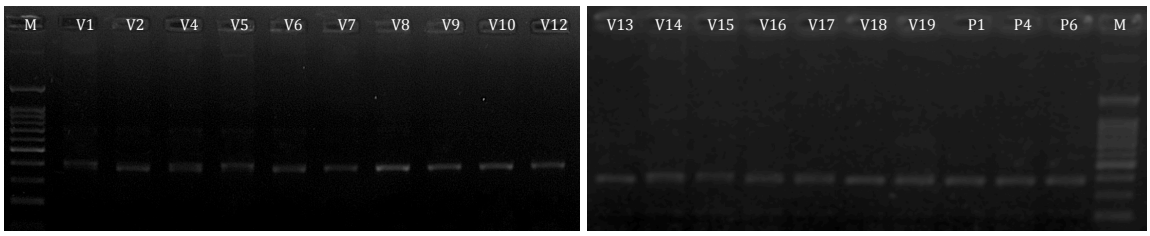


Figure 10 - Gel electrophoresis analysis of PCR products of *CPSE* exon1-exon2 region (~416 bp) from DNA extracted from blood samples of 20 male dogs (M-molecular marker).

A similar result, where it was possible to observe the expected 575 bp fragment in all DNA samples (Figure 11), was achieved for CPSE_Ex3-4 set of primers.

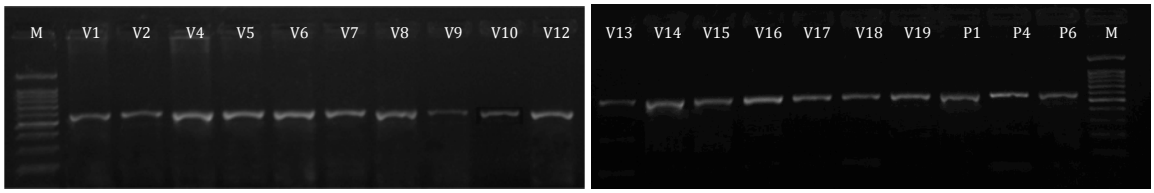


Figure 11 - Gel electrophoresis analysis of PCR products of *CPSE* exon3-exon4 region (~575 bp) from DNA extracted from blood samples of 20 male dogs (M-molecular marker).

In order to evaluate the expression of these two regions of *CPSE* gene in animals presenting different prostatic conditions, a RT-PCR using RNA extracted from prostatic fluid samples was performed. Initially, PCR amplification of a housekeeping gene (GAPDH) was optimized and the PCR products were analyzed by electrophoresis on a 1,5% agarose gel. Using the primers GAP_Cf_F and GAPDH_Ctrl_R, the expected 176 bp fragment was seen in all cDNA samples (Figure 12).

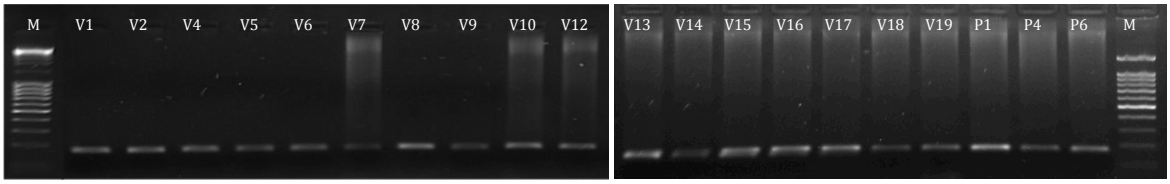


Figure 12 – Gel electrophoresis analysis of RT-PCR products of *GAPDH* gene (~176 bp) from RNA extracted from prostatic fluid samples of 20 male dogs (M-molecular marker).

Following the gel electrophoresis analysis of RT-PCR products of the housekeeping gene, the two regions of *CPSE* gene were amplified by PCR and the amplicons were analyzed by electrophoresis. Using CPSE_Ex1-2 set of primers, the expected 218 bp fragment was seen in all cDNA samples with the exception of V1, V2 and V18 cDNA samples (Figure 13).

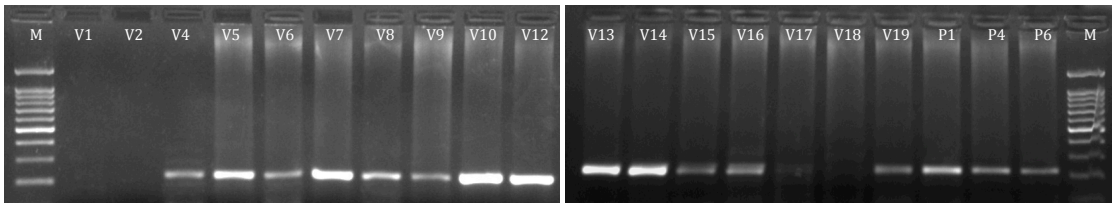


Figure 13 - Gel electrophoresis analysis of RT-PCR products of *CPSE* exon1-exon2 region (~218 bp) from RNA extracted from prostatic fluid samples of 20 male dogs (M-molecular marker).

Use of CPSE_Ex3-4 set of primers revealed the expected 400 bp fragment in all cDNA samples with the exception of V1, V2, V4 and V18 cDNA samples (Figure 14).

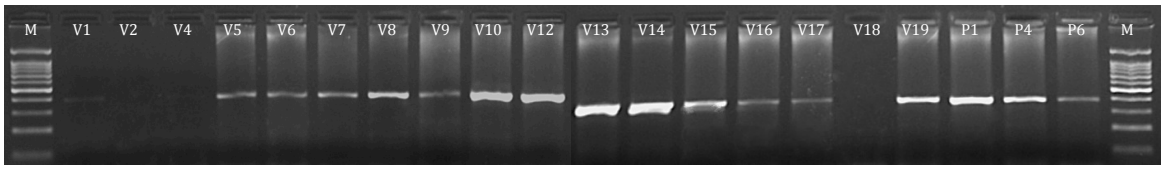


Figure 14 - Gel electrophoresis analysis of RT-PCR products of *CPSE* exon3-exon4 region (~400 bp) from RNA extracted from prostatic fluid samples of 20 male dogs (M-molecular marker).

In addition to the analysis of these two regions of *CPSE* gene, another candidate gene - *PCA3* - was also analyzed at DNA and RNA levels. The genome electronic databases (NCBI, Ensembl and others) do not have information about *PCA3* gene in dogs. Consequently, it was of interest to determine its locus in the canine genome. Using BLAST database for *Canis familiaris*, *PCA3 Homo sapiens* sequence (AF103908.1) was aligned with canine chromosome 1 (12.198.500 to 12.204.228 positions) presenting 79% of identity (1565/1975). To enlarge the knowledge of this genomic region, eight specific sets of primers were selected (Annex IV) and PCR was performed using the DNA of a normal control dog. The amplicons were analyzed by electrophoresis on a 1,5% agarose gel and eight fragments with the expected length (600, 632, 605, 645, 602, 772, 814 and 511 bp respectively) were observed (Figure 15).

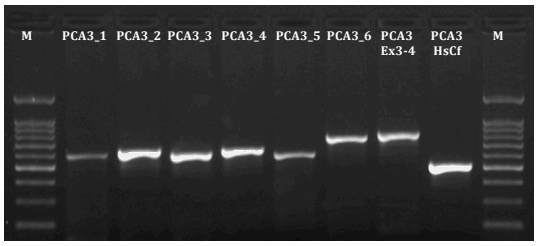


Figure 15 – Gel electrophoresis analysis of PCR products of *Canis familiaris* chromosome 1 identified region from DNA extracted from blood of normal control dog using eight specific sets of primers (M-molecular marker).

PCR products observed in Figure 15 were purified and sequenced in both directions and the output sequences were analyzed and edited. The next step consisted in the prevision of the different exonic regions in the assembled sequence, comparing with the existing information on *Homo sapiens*. The different human *PCA3* exonic sequences were aligned with the assembled sequence and the sequence identity (%) was calculated by the number of identical nucleotides in the alignment divided by the length of human exon sequence (Table II), following the strategy described by Clarke and colleagues (2009).

Table II – Sequence identity (%) of PCA3 exons between human and dog.

	Exon 2 (165bp)	**Exon 3** (183bp)	**Exon 4a** (537bp)	**Exon 4b** (1061bp)	**Exon 4c** (1856bp)	**Whole exon 4** (3454bp)
Assembled sequence	69	77	75	78	77	77

Additionally, *PCA3* gene expression was analyzed in dogs presenting different prostatic conditions using the eight specific sets of primers (Annex IV). PCR was performed using the cDNA of all animals and the PCR products were analyzed by electrophoresis on a 1,5% agarose gel. The expected fragments were not observed using PCA3_1 (Figure 16) neither the other sets of primers, indicating a lack of expression of *PCA3* for all cDNA samples.

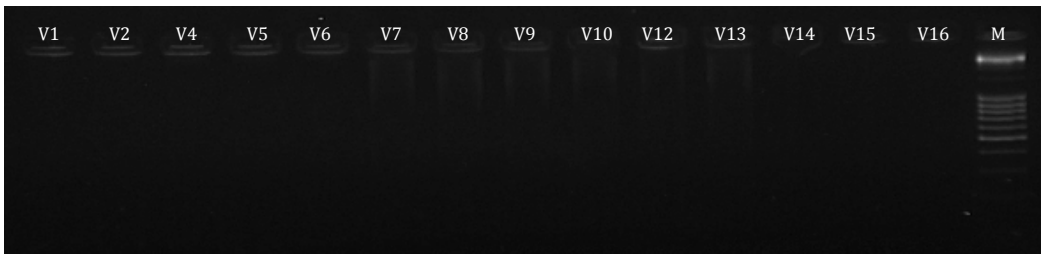


Figure 16 –Gel electrophoresis analysis of RT-PCR products of *PCA3* gene from RNA extracted from prostatic fluid of 14 dogs (V1-V16) using PCA3_1 set of primers (M-molecular marker).

Another important purpose of the present work was to conduct a molecular analysis of two regions (5´-UTR region to exon 2 and exon 5 to 3'UTR region – Annex V) of dog *IL-6* gene, in order to identify genetic variations and verify its association with different prostatic disorders.

After selecting specific primers, these two *IL-6* gene regions were amplified by PCR and the amplicons were analyzed by electrophoresis, revealing the expected 431bp (Figure 17) and 630bp (Figure 18) fragments in all DNA samples.

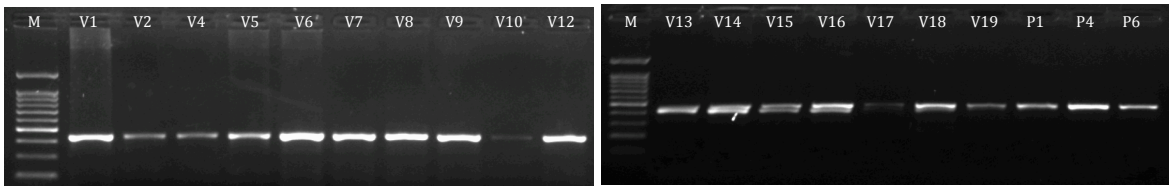


Figure 17 - Gel electrophoresis analysis of PCR products of *IL-6* 5´-UTR to exon 2 region (~431 bp) from DNA extracted from blood samples of 20 male dogs (M-molecular marker).

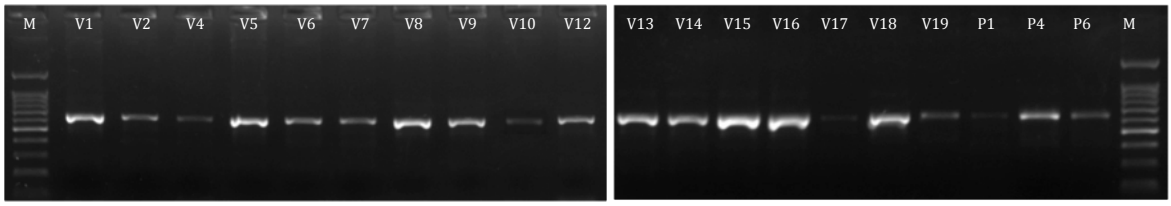


Figure 18 - Gel electrophoresis analysis of PCR products of *IL-6* exon 5 to 3'UTR region (~630 bp) from DNA extracted from blood samples of 20 male dogs (M-molecular marker).

All amplified fragments were purified and sequenced in both directions. The output sequences were analyzed, edited and aligned (Annex VII and Annex VIII). In the first fragment (5´-UTR region to exon 2) no genetic variations were detected. On the other hand, concerning the fragment from exon 5 to 3'-UTR region, two single nucleotide variations were identified (Table III). The genetic variation located in the coding region of exon 5 (I/5_g105G>A) was characterized by the transition of guanine to adenine, which affects the encoded amino acid (arginine to glutamine). Additionally, a transition of guanine to adenine (I/5_g440G>A) was also detected in the untranslated region of exon 5. The frequencies of genotypes were 88,9% to GG and 11,1% to GA for I/5_g105G>A variation and 94,4% to GG and 5,6% to GA regarding I/5_g440G>A variation. The genotype AA was not observed in any of these genetic variations.

Table III – Location and description of *IL-6* genetic variations identified in the present study.

Gene	Variation	Location	Codon	Amino acid
IL-6	I/5_g105G>A	Exon 5	CGG/CAG	Arg (R)/ Gln(D)
	I/5_g440G>A	Exon 5	UTR	-

In the last approach of the present work a meta-analysis of case-control studies was performed in order to derive a more precise estimation of the relationship between *IL-6* polymorphisms and human PCa risk.

The initial PubMed and HighWire search identified a total of 77 entries but only seven met the criteria to be included in the present meta-analysis. Additionally, one study of interest was found in the references of the seven previously selected studies. Consequently, eight eligible studies (Sun *et al.,* 2004; Michau *et al.,* 2006; Bao *et al.,* 2008; Moore *et al.,* 2009; Pierce *et al.,* 2009; Wang *et al.,* 2009; Zabaleta *et al.,* 2009; Dossus *et al.,* 2010,) were included (Figure 19).

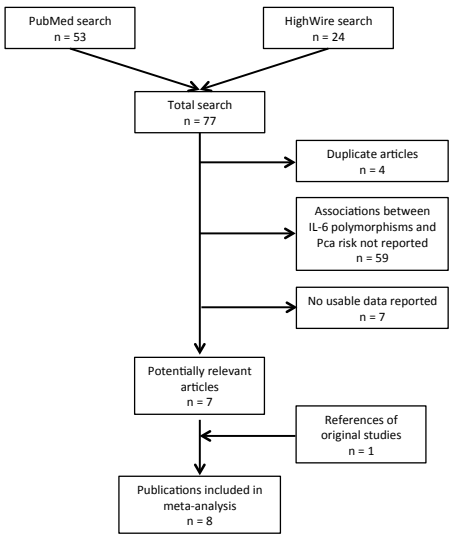


Figure 19 - Flow diagram showing the selection process of the publications included in the present meta-analysis.

The main characteristics of studies are summarized in Table IV. There were seven studies composed of 11277 PCa cases and 13672 controls concerning the *IL6* -174G>C polymorphism and four studies composed of 1941 PCa cases and 3357 controls regarding the *IL-6* -636G>C polymorphism.

Table IV - Main characteristics of studies included in the present meta-analysis.

Study	Ethnicity	Study design	Number of Cases (GG/GC/CC)	Number of Controls (GG/GC/CC)
***IL-6* -174G>C**				
Dossus *et al.*, 2010	Caucasian	Case/Control	3594/3218/1125	3832/3402/1274
Michau *et al.,* 2006	Caucasian	Case/Control	170/223/91	230/293/90
Moore *et al.*, 2009	Caucasian	Case/Control	191/485/281	196/401/250
Pierce *et al.*, 2009	Caucasian	Case/ Control	48/96/31	696/901/336
Pierce *et al.*, 2009	African	Case/ Control	34/5/1	250/48/2
Sun *et al.*, 2004	Caucasian	Case/Control	350/667/328	205/389/167
Wang *et al.*, 2009	Caucasian	Case/Control	91/116/43	84/128/40
Zabaleta *et al.*, 2009	Caucasian	Case/Control	19/34/21	126/163/112
Zabaleta *et al.*, 2009	African	Case/Control	10/2/3	41/10/6
***IL-6* -636G>C**				
Bao *et al.*, 2008	Asian	Case/Control	47/39/50	28/27/65
Pierce *et al.*, 2009	Caucasian	Case/Control	156/19/0	1740/192/2
Pierce *et al.*, 2009	African	Case/Control	37/2/1	253/46/1
Sun *et al.*, 2004	Caucasian	Case/Control	1226/109/2	675/74/4
Wang *et al.*, 2009	Caucasian	Case/Control	233/19/1	225/25/0

In the overall analysis, *IL-6* -174G>C polymorphism was not associated with PCa risk. As indicated in Table V, the *IL-6* -174G>C genotypes were not statistically significantly associated with PCa risk in all genetic models. However, in the stratified analyses by ethnicity, the CC and CG genotypes were found to be associated with increased risk of PCa (P=0,015; OR=1,442) in the dominant genetic model of African descents.

Table V - Analysis of *IL-6* -174G>C polymorphism and PCa risk.

Genetic model	**Main effects of *IL-6* -174G>C polymorphism and prostate cancer**		
(number of studies)	***OR (95% CI)***	***P-value***	$P_{Heterogeneity}$
Overall (9)			
C vs G	1,009 (0,971 – 1,048)	0,657	0,337
CC vs GG	1,012 (0,937 – 1,093)	0,764	0,219
GC vs GG	1,030 (0,972 – 1,091)	0,312	0,261
CC vs GG+GC	0,992 (0,925 – 1,064)	0,831	0,318
CC+CG vs GG	1,021 (0,968 – 1,078)	0,446	0,270
Ethnic groups			
African (2)			
C vs G	1,209 (0,651 – 2,245)	0,547	0,520
CC vs GG	1,306 (0,889 – 1,919)	0,174	0,861
GC vs GG	0,780 (0,333 – 1,825)	0,566	0,945
CC vs GG+GC	1,023 (0,738 - 1,418)	0,892	0,998
CC+CG vs GG	1,442 (1,075 – 1,935)	**0,015**	0,732
Caucasian (7)			
C vs G	1,008 (0,970 -1,047)	0,683	0,216
CC vs GG	1,001 (0,925 – 1,083)	0,978	0,178
GC vs GG	1,032 (0,974 – 1,093)	0,293	0,141
CC vs GG+GC	0,991 (0,922 – 1,065)	0,803	0,160
CC+CG vs GG	1,009 (0,955 – 1,066)	0,748	0,634

Concerning the *IL-6* -636G>C polymorphism analyses (Table VI), the C allele was significantly associated with decreased risk of PCa when tested in the overall analysis (P=0,002; OR=0,735) and in the stratified analyses by ethnicity of Asian descents (P=0,001; OR=0,553).

Similarly, a significant decreased risk was observed for the CC genotype in the homozygous comparison and in the recessive genetic model in the overall analysis (P=0,021, OR=0,532; P=0,011, OR=0,547 respectively) and in the stratified analyses by ethnicity of Asian descents (P=0,010; OR=0,458; P=0,005; OR=0,492 respectively). The dominant genetic model (CC+GC vs GG) was also associated with a decreased risk for PCa in Asian population (P=0,050; OR= 0,576) and in overall analysis (P=0,024; OR=0,780). Additionally, heterogeneity was not detected.

Table VI - Analysis of *IL-6* -636G>C polymorphism with PCa risk.

Genetic model	**Main effects of *IL-6* -636 G>C polymorphism and prostate cancer**		
(number of studies)	*OR (95% CI)*	*P-value*	$P_{Heterogeneity}$
Overall (5)			
C vs G	0,735 (0,608 – 0,889)	**0,002**	0,269
CC vs GG	0,532 (0,312 – 0,908)	**0,021**	0,203
GC vs GG	0,838 (0,669 – 1,050)	0,124	0,503
CC vs GG+GC	0,547 (0,344 – 0,869)	**0,011**	0,193
CC+CG vs GG	0,780 (0,628 – 0,968)	**0,024**	0,436
Ethnic groups			
African (1)			
C vs G	0,605 (0,212 – 1,726)	0,348	1,000
CC vs GG	6,838 (0,419 – 111,683)	0,177	1,000
GC vs GG	0,297 (0,069 – 1,276)	0,103	1,000
CC vs GG+GC	7,667 (0,470 – 125,049)	0,153	1,000
CC+CG vs GG	0,436 (0,129 – 1,474)	0,182	1,000
Asian (1)			
C vs G	0,553 (0,387 – 0,789)	**0,001**	1,000
CC vs GG	0,458 (0,253 – 0,831)	**0,010**	1,000
GC vs GG	0,861 (0,437 – 1,695)	0,664	1,000
CC vs GG+GC	0,492 (0,298 – 0,811)	**0,005**	1,000
CC+CG vs GG	0,576 (0,332 – 1,000)	**0,050**	1,000
Caucasian (3)			
C vs G	0,836 (0,664 – 1,053)	0,128	0,500
CC vs GG	0,628 (0,163 – 2,415)	0,499	0,295
GC vs GG	0,859 (0,674 – 1,095)	0,220	0,510
CC vs GG+GC	0,637 (0,166 – 2,450)	0,512	0,298
CC+CG vs GG	0,844 (0,664 – 1,072)	0,165	0,513

Chapter V – Analysis and Discussion of Results

Chapter V – Analysis and Discussion of Results

The main function of prostate gland is to produce prostatic fluid that ensures the nourishment and proper motility of sperm. Disease conditions affecting this gland are very common in older men and can include BPH, prostatitis and PCa.

The different prostatic diseases present multifactorial aetiology and the mechanisms involved in their predisposition, development and progression are still unclear. In this context, studies using animal models that can inter-relate several candidate genes with different prostatic conditions are essential for the overall understanding of the molecular mechanisms involved in these pathologies.

According to LeRoy and Northrup (2009), *Canis familiaris* is a good animal model for the study of human prostatic diseases, due to the morphological and functional similarities between the canine and human prostate glands. Additionally, dogs and humans share the same environment, minimizing the influence of environmental factors (Winkler *et al*., 2005) and dogs are large animal models, which make feasible imaging, diagnostic studies and therapeutic interventions (Waters and Bostwick, 1997).

Combining the existing information on human prostatic diseases with the usefulness of *Canis familiaris* as an animal model, the present work accomplished different genetic approaches that were performed in a population of 20 adult male dogs presenting different prostatic conditions.

In order to define the most appropriate strategies, it was important to have a final clinical diagnosis of the population. This process was laborious because it involved a detailed examination of the physical condition of the animal as well as a detailed examination of the prostate and testicles.

DRE is considered the best method for the physical examination of the prostate gland but its effectiveness depends on the ability of the veterinarian to detect abnormalities (Smith, 2008). In the present work, DRE procedures were performed in HVUTAD by a team of professional veterinarians with the purpose of obtaining a solid interpretation of results. Prostate size, consistency, limits, sensivity, symmetry and the presence of nodules were examined to find out abnormal conditions or signs of prostatic disease. In normal conditions, the prostate was smooth and symmetrical, DRE procedure did not cause pain and no abnormal nodules were detected. On the other hand, pathological states were associated with abnormalities in these features.

DRE, ultrasonography and cytological examination were essential procedures to obtain the definite diagnosis in veterinary clinical context. Similarly, the optimization of collection and processing protocols were crucial to obtain the required biological samples in order to perform different genetic analysis.

The DNA extraction was performed using the QuickGene DNA Whole Blood kit S (Fujifilm) and the RNA was extracted using QIAmp viral kit (Qiagen) with some specific changes that were previously optimized. Good concentrations of DNA (102,6 - 198,5 ng/µl) and RNA (64,8 - 169,2 ng/µl) were achieved, confirming that these optimized kits are appropriated for nucleic acids extraction.

Following the extraction procedures, different genetic approaches were implemented to contribute to a deeper knowledge of the molecular mechanism involved in canine prostatic disorders; to the development of efficient strategies to diagnose these pathologies in veterinary clinical context and eventually, to validate the applicability of the canine model in genetic studies of human prostatic disorders.

5.1 – CPSE as a diagnostic biomarker for prostatic diseases in *Canis familiaris*

Benign prostatic hyperplasia (BPH) is the most common disease of canine prostate gland, affecting 95% of dogs with more than nine years of age (Gobello and Corrada, 2002). Clinical signs include sanguineous fluid dripping from the urethra, intermittent urethral discharge, tenesmus associated with defecation, pain, lameness and behavioural problems (LeRoy and Northrup, 2009). Due to the high prevalence of this condition, it is necessary to develop/improve non-invasive diagnostic methods for veterinary clinical context.

CPSE represents 90% of the secretory products of canine prostatic epithelial cells, is highly dependent on androgens, is member of kallikrein gene family (Chapdelaine *et al*., 1988) and has approximately 60% similarity with human PSA at amino acid level (Lundwall and Lilja, 1987).

Bell and colleagues (1995) measured CPSE serum concentration in 25 dogs presenting clinical signs of BPH and in 20 normal control dogs and their results demonstrated that CPSE serum concentration in dogs with BPH was significantly higher (mean concentration = 189,7 ng/mL) compared with normal intact dogs (mean concentration = 41,8 ng/mL). Furthermore, Gobello and colleagues (2002) reviewed the most relevant clinical features of the main genital markers in the male dog and they concluded that CPSE is a good marker of canine prostate gland secretion and therefore a promising biomarker for BPH detection. Recently, Virbac© provided an immunoassay (Odelis®CPSE) that, with a simple blood test, measures the CPSE plasma concentration. In order to test the validity of this assay in canine prostatic disorders detection, Odelis®CPSE was performed for all plasma samples and the obtained results were analyzed taking into consideration the clinical and cytological diagnoses (Table I).

Odelis®CPSE results were in accordance with the clinical and cytological diagnoses for all samples with the exception of DPV8 and DPV14. Concerning the obtained results, these animals were associated with BPH condition; however, according to the clinical and cytological

diagnoses, DPV8 presented signs of prostatitis and DPV14 was diagnosed as a squamous metaplasia case. The epithelial cells observed in the cytological examination of DPV8 were scarce and had poorly preserved morphology. The repetition of this analysis was recommended but was not performed due to the difficulty in obtaining permission of the owner to execute another manipulation of the animal. On the other hand, squamous metaplasia is associated with excessive serum estrogen concentrations as a result of exogenous estrogen administration or estrogen-secreting Sertoli tumors (Gobello and Corrada, 2002); consequently the high estrogen levels might affect CPSE measurement and give unexpected results.

As expected, CPSE plasma concentrations were higher in pathological conditions than in normal control dogs, indicating that CPSE production and secretion may be increased in pathological conditions. Nevertheless, it was not possible to differentiate dogs with BPH from those with other prostatic disorders. In this context and due to the small number of animals presenting prostatitis, squamous metaplasia and BPH plus prostatitis, only the BPH and normal cases were statistically tested. CPSE concentrations of these two groups were compared using SPSS software (independent samples T-test). CPSE plasma concentration was elevated in dogs with BPH (mean concentration = 160,75 ng/ml) compared with normal control dogs (mean concentration = 25,73 ng/ml) (Figure 9). These two groups were significantly different (P-value=0,001) confirming that dogs presenting signs of BPH have higher CPSE concentration than normal dogs.

CPSE concentrations measured in BPH cases were higher (69,26 - 266,37 ng/mL) than the expected values (54–67 ng/mL) indicated in Odelis®CPSE assay brochure, so further studies are necessary to assess the validity of this test in veterinary clinical context.

5.2 – Genetic analysis of *CPSE*, *PCA3* and *IL-6* genes in *Canis familiaris*

Recent genetic analyses of human prostatic diseases were the starting point for applying comparative genomic tools in order to reach information about canine prostatic diseases and evaluate the potential role of candidate genes in deciphering the main differences between the different forms of prostate condition. A candidate gene is a gene that is suspected to have a relevant role in a particular process or disease condition.

The choice of the suitable candidate gene is determinant for the success of the genetic analysis. *CPSE* was chosen due to its similarity to human *PSA* and based on the fact that it seems to be a promising diagnostic tool in differentiating BPH from the other prostatic conditions. *PCA3* was analyzed due to its central role as a biomarker for human PCa detection and to the absence of information about this gene in *Canis familiaris*. *IL-6* gene was selected because

several studies have demonstrated that this gene is involved in the regulation of cell growth, differentiation and survival, which may influence prostatic diseases predisposition.

5.2.1 – *CPSE* gene expression in different canine prostatic conditions

The canine *CPSE* gene is located on chromosome 1, spans 5510 bp and consists in five exons (GenBank: M63669.1; Gene ID: 403967, updated on 22-Dec-2011). Specific primers to two regions of *CPSE* gene (exon1-exon2 and exon3-exon4) were selected and these regions were analyzed at DNA and RNA levels.

PCR conditions were optimized using DNA extracted from the buffy coat layer of blood samples and the PCR products were analyzed by electrophoresis on a 1,5% agarose gel.

Concerning the *CPSE* exon1-exon2 region, the expected 416bp fragment was seen in all DNA samples (Figure 10), confirming the appropriated amplification of this region. The same results were obtained for *CPSE* exon3-exon4 region by the detection of the expected 575 bp fragments in all DNA samples (Figure 11).

Gene expression studies play a central role in biomedical research (Vermeulen *et al*., 2011). RT-PCR can provide a good reflection of an organism's gene-expression profile so, in order to evaluate *CPSE* gene expression pattern in dogs presenting different prostatic diseases, this technique was performed using RNA extracted from prostatic fluid samples. Reliable extraction of equal amounts of non-degraded RNA from each sample; consistent reverse transcriptase efficiency resulting in equal amounts of cDNA in all samples; adequate primer specificity and the presence of inhibitors in samples are drawbacks that affect the interpretation of RT-PCR results (Turabelidze *et al.*, 2010). To overcome these obstacles and normalize the results, an analysis of a housekeeping gene expression was performed.

Housekeeping genes are genes involved in basic and ubiquitous cellular functions (Turabelidze *et al.*, 2010). A suitable housekeeping gene should be expressed in the tissue of interest at stable levels between samples and under different biological contexts. Beta actin, glyceraldeyde-3-phosphate dehydrogenase (*GAPDH*), ribosome small subunit and ubiquitin C are housekeeping genes commonly used for RT-PCR validation. Based on the results of a previous study, *GAPDH* was chosen as a control for the quality of cDNA synthesis. PCR amplification of *GAPDH* was performed using previously selected primers (GAP_Cf_F and GAPDH_Ctrl_R) and PCR products were analyzed by electrophoresis on a 1,5% agarose gel. The expected 176 bp fragment was seen in all cDNA samples (Figure 12), confirming the good quality of these samples for gene expression studies.

Following the analysis of *GAPDH* RT-PCR products, two regions of *CPSE* gene were amplified by PCR and the amplicons were analyzed by electrophoresis. Figures 13 and 14 show that for V1,

V2 and V18 dogs no expression of these *CPSE* regions was detected; *CPSE* exon3-exon4 region was detected for V7 dog but no expression was detected concerning the *CPSE* exon1-exon2 region. For V4 animal *CPSE* exon1-exon2 fragment was obtained but no amplification was detected for *CPSE* exon3-exon4 region. According to the clinical diagnosis V1, V2 and V4 present signs of BPH, V17 signs of prostatitis and V18 has a normal prostate gland. According to these results, it was not possible to associate the expression profile of this gene with a specific prostatic condition. Due to the findings of Bell and colleagues (1999) that revealed that CPSE serum concentration in dogs with BPH is higher than in normal control dogs, this result was not expected. As referred before, the *GAPDH* expression analysis revealed that the cDNAs were suitable for gene expression studies. In this context, the lack of amplification may be due to mutations in the primer region or to the lower concentration of CPSE transcripts in these samples. Future prospects include a detailed description of this gene in a larger population, involving the detection of genetic variants, association studies and the use of quantitative real time PCR to assess the possible differential expression of this gene in different prostatic conditions.

5.2.2 – Characterization of canine *PCA3* gene

The significant progress in the investigation of biomarkers for PCa screening and detection has lead to the development of a novel non-invasive urine-based assay (Makarov and Loeb, 2009). The urinary *PCA3* test has shown promising results for human PCa detection and in counseling and confirming biopsy indications (Auprich *et al.*, 2011).

The *PCA3* gene is a non-coding RNA, highly overexpressed in malignant prostate tissue compared with benign and normal tissues (Chun *et al*., 2009; Schilling *et al*., 2009; Ruiz-Aragon and Márquez-Peláez, 2010). In order to understand the importance of this gene in canine prostatic diseases, the *PCA3* locus in the canine genome was explored.

Searching the genome electronic databases (NCBI and Ensembl), no information was found concerning the *PCA3* gene in *Canis familiaris*. Accordingly, a BLAST search was performed and a significant alignment (79% of identity) was achieved. Analyzing the results in detail, the query sequence (AF103908.1) aligned with a region similar to a *BNIP2* motif-containing molecule at the carboxy terminal region 1 (also known as *BMCC1* gene), located in *Canis familiaris* chromosome 1 (12.198.500 to 12.204.228). This result was consistent with the new genomic structure for human *PCA3* gene proposed by Clarke and colleagues (2009). These findings indicate a similarity with humans, which validate this approach and emphasize the fact that dogs are good models for studying prostatic diseases.

To characterize this canine genomic region, eight sets of primers were selected and PCR conditions were optimized using the DNA of a normal control dog. The amplicons were analyzed by electrophoresis on a 1,5% agarose gel and eight fragments with the expected length were observed (Figure 15). Subsequently, PCR products were purified and sequenced. The sequencing results were visualized, analyzed and edited to obtain the final sequence. The assembled dog *PCA3* gene was aligned with *Canis familiaris* chromosome 1 (12.198.500 to 12.204.228) and a complete alignment (100% of similarity) was achieved. The next step consisted in the prediction of exon sites.

There are conserved nucleotide sequences present at the junctions of introns and exons in which the splicing reactions take place. Of particular importance are the GT nucleotides in the 5' end and the AG nucleotides in the 3' end of an intron. Another invariant site is an A residue labeled "branch point" that is located between 15 and 45 nucleotides upstream of the 3' splice site (Griffiths *et al.*, 2008). Several bioinformatic programs were applied to predict exons and to find open reading frames (ORFs), however no consistent results were accomplished.

Using the human *PCA3* sequence as the reference, the assembled canine *PCA3* sequence was aligned with each human PCA3 exon and the exonic identities were calculated by the number of identical nucleotides in the alignment divided by the length of human exon sequence (Table II).

The exon 4b appears to be the most identical region (78% of identity) and the exon 2 the less identical region (69% of identity) between human and dog. Nevertheless, this strategy did not give consistent results because it did not take into account the conserved nucleotide sequences and the gaps were excluded in the calculation of sequence identity.

Another mean of identifying exons and ORF´s is through the analysis of mRNA expression. The cDNA sequence is extremely valuable because it is complementary to the mature mRNA (no introns), which facilitates the identification of the exons and introns of a gene. Consequently, PCR was performed using the cDNA of all animals and the PCR products were analyzed by electrophoresis on a 1,5% agarose gel. The expected fragments were not observed using PCA3_1 (Figure 16) neither the other primers sets, indicating a lack of expression of *PCA3* for all cDNA samples. In the analyzed population, none of the animals presented signs of PCa so, these results may suggest that *PCA3* gene expression is only associated with neoplasic prostatic condition. To confirm this possibility, PCA3 expression should be analyzed in a population involving a significant number of animals with PCa.

5.2.3 – *IL-6* gene variants in *Canis familiaris*

Genetic research has contributed to a better understanding of prostatic diseases and has shown that genes involved in the inflammation pathway have been associated with cancer risk

(Sutcliffe and Platz, 2007; Xu *et al*., 2011).

IL-6 gene encodes a multifunctional glycoprotein that is involved in the regulation of inflammation (Akira *et al.*, 1993). Polymorphisms in *IL-6* gene may result in an altered level of expression, and hence this gene must be considered an important candidate for the analysis of prostatic disorders susceptibility. There are 187 SNP's described in human *IL-6* gene (NCBI database) and some of them have been associated with PCa susceptibility. On the other hand, there are only five SNP's described in canine *IL-6* gene and none of them was associated with prostatic diseases susceptibility.

The present work accomplished the molecular analysis of two *IL-6* gene regions (5´-UTR region to exon 2 and exon 5 to 3'UTR region) in order to identify new *IL-6* gene variations and verify their association with different prostatic disorders.

PCR conditions were optimized and the amplicons were analyzed by electrophoresis, revealing the expected 431bp (Figure 17) and 630bp (Figure 18) fragments in all DNA samples. All amplified fragments were sequenced allowing the identification of two genetic variations: I/5_g105G>A and I/5_g440G>A (Table III).

The genetic variation located in the coding region of exon 5 (I/5_g105G>A) was characterized by the transition of guanine to adenine, affecting the encoded amino acid (arginine to glutamine). In 2011, Morinha and colleagues identified and characterized I/5_g105G>A variant in an association study between *IL-6* gene variants and canine periodontal disease. They used the Sorting Tolerant From Intolerant (SIFT) algorithm to predict if the amino acid substitution caused by I/5_g105G>A variant affects IL-6 protein function and they concluded that this gene variant probably has damage effects (SIFT score = 0,02). Based on the SWISS-MODEL structure homology-modeling server they predicted the canine IL-6 structures and observed that this variation is located in an exposed region. They also compared the predicted structures with human IL-6/IL-6α-receptor/gp130 complex and found that I/5_g105G>A may have important roles in the functional association of IL-6/IL-6α-receptor and the protein-protein interactions. Finally, they predicted the functional interactions of the dog IL-6 protein and demonstrated that IL-6 molecule is integrated in a complex network involving multiple protein and metabolic pathways. As a consequence of the aforementioned findings, the I/5_g105G>A variation is considered of special interest in future analyses.

Additionally, a transition of guanine to adenine (I/5_g440G>A) was also detected in the untranslated region of exon 5. SNPs present in noncoding regions may have effects on gene expression by affecting regulatory elements (Goto *et al.*, 2001). Future studies are necessary to confirm if I/5_g440G>A variation affects the levels of gene expression, influencing the susceptibility to develop a specific prostatic disease.

The reduced number of animals in the analyzed population was not significant for the case-

control analysis. The genetic variation located in the coding region of exon 5 (I/5_g105G>A) was identified in two animals: one with BPH signs (V7) and other with prostatitis (V8), representing only 11,1% of the population. The I/5_g440G>A variation was only detected in a normal control dog (V9), representing 5,6% of the population. In this context, these two genetic variations were not correlated with susceptibility to any prostatic condition in dogs.

Future case-control studies involving the identification of other gene variants and the analysis of their interaction in a larger population may help to establish profiles of susceptibility for the different prostatic disorders.

5.4 – *IL-6* gene variants and human PCa susceptibility

Another main goal of the present work was the analysis of the existing scientific publications related to *IL-6* gene variants and human PCa susceptibility.

Multiple studies investigated the role of cytokine genes polymorphisms in the etiology of cancer of various organs including prostate, breast, colorectal, gastric, lung, and so on (Howell and Rose-Zerilli, 2007; Sutcliffe and Platz, 2007; Xu *et al*., 2011). However, the results of these studies remain conflicting.

Due to the important roles of IL-6 in different physiologic and pathophysiologic processes, *IL-6* polymorphisms may modulate cancer risk (Tan *et al*., 2005). Fishman and colleagues (1998) reported that -174 G<C *IL-6* polymorphism influence the binding of the glucocorticoid receptor and thus repress transcriptional activation. Their results demonstrated that the -174 C allele had a reduced ability to bind glucocorticoid receptor and was associated with low plasma IL-6 levels. In 2011, Ahn and colleagues verified that the GG and GC genotypes of *IL-6* -636 G>C polymorphism were associated with higher IL-6 serum levels than the CC genotype. An elevated serum IL-6 level has been correlated with worse clinical outcomes and PCa risk (Kishimoto, 2005; Terakawa *et al*., 2009). According to these findings, the presence of C allele in IL-6 -174 G<C and -636 G>C polymorphisms might reduce PCa risk.

Multiple studies have investigated the association between these two common polymorphisms in *IL-6* promoter region and PCa risk (Sun *et al.,* 2004; Michau *et al.,* 2006; Bao *et al.,* 2008; Moore *et al.,* 2009; Pierce *et al.,* 2009; Wang *et al.,* 2009; Zabaleta *et al.,* 2009; Dossus *et al.,* 2010). Whereas some studies revealed no significant increased or decreased PCa risk among men carrying *IL-6* sequence variants (Sun *et al.,* 2004; Michau *et al.,* 2006; Moore *et al.,* 2009; Pierce *et al.,* 2009; Wang *et al.,* 2009; Zabaleta *et al.,* 2009; Dossus *et al.,* 2010), Bao and colleagues (2008) found a significant association between GG and GC genotypes of *IL-6* -636G>C polymorphism and increased risk of PCa in Asian individuals.

To our knowledge, the present meta-analysis is the first evaluation of the association between *IL-6* -174G>C and -636 G>C promoter polymorphisms and PCa risk. In the overall *IL-6* -174G>C analysis, no significant differences in both allele and genotype frequencies between PCa cases and healthy controls were observed (Table V). These results were expected because most individual studies included in the present meta-analysis also found no association between this polymorphism and PCa risk. Nevertheless, in the stratified analyses by ethnicity, the dominant genetic model was found to be associated with increased risk in African descents (Table V), suggesting that CC and CG African individuals may have a higher susceptibility to PCa than those with GG genotype. Present data supports that ethnic differences across populations may explain the higher PCa incidence observed in populations of African descent, suggesting that ancestral genetic factors may increase PCa susceptibility.

Regarding the *IL-6* -636 G>C polymorphism, the CC and GC genotypes were significantly associated with decreased risk of PCa in the homozygous comparison and in the dominant and recessive genetic models in the overall analysis (Table VI). The stratified analyses by ethnicity demonstrated that Asian individuals with CC/GC genotypes might have a slight decrease in susceptibility compared with individuals with GG genotype (Table VI). The presence of the C allele seems to have a protective effect on Pca susceptibility. These results were consistent with the findings of Bao and colleagues (2008). These authors suggested that Asian individuals with GG or GC genotypes have higher PCa risk than those with CC genotype.

The present meta-analysis reinforced the possible association between *IL-6* promoter polymorphisms and PCa susceptibility. However, this work was limited by the few number of studies that have assessed the relationship between *IL-6* promoter region polymorphisms and PCa risk and the fact that SNP's in cytokine promoter region represent only one piece of the very complex nature of PCa. Consequently, in order to derive a more comprehensive understanding of the association between *IL-6* genetic variants and PCa risk, further studies based on the interactions of PCa susceptibility genes and environment factors are warranted.

Chapter VI – Conclusions and Future Prospects

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The present work involved different strategies to analyse a population of 20 adult male dogs presenting different prostatic conditions, in order to enlarge the knowledge regarding the canine prostatic diseases. It aimed to contribute to the improvement of efficient diagnosis tools and to the implementation of the dog as an animal model in the study of the genetic basis of human prostatic disorders.

In this perspective, the main conclusions were:

- Regarding the application of Odelis®CPSE test, when comparing the normal animals with the BPH group, significant differences were observed. This result confirmed that dogs presenting signs of BPH have higher plasma CPSE concentration than dogs with a normal prostate gland. This confirmation is an important achievement and indicates that this test has applicability in the clinical context, in order to differentiate normal dogs from dogs with BPH. Odelis®CPSE is a simple test that provides a rapid evaluation of CPSE plasma concentration and contributes to an early non-invasive diagnosis of canine prostatic diseases. Our results showed a limitation of this test in what concerns the differentiation between BPH and other prostatic diseases (as prostatitis, BPH with prostatitis and squamous metaplasia). This restriction points out for the need to continue the investigation of biomarkers with better performance. In this context, comparative genomic tools are essential to reach information about canine prostatic pathologies and evaluate the potential role of candidate genes in deciphering the main differences between the different forms of prostatic diseases.

- *CPSE* was evaluated at the mRNA level by reverse transcription PCR. The absence of amplification of CPSE transcripts in some animals was not indicative of a possible association with a specific prostatic condition. In fact, among the three animals in which the two fragments were not amplified, two were diagnosed with BPH and one had a normal prostate gland. Further association studies involving the use of quantitative real time PCR should be determinant to associate a differential expression of this gene with a specific prostatic condition.

- Exploring the *PCA3* locus in the canine genome, the *PCA3* assembled gene sequence presented 79% of identity with human *PCA3* sequence. This result emphasizes the fact that dogs might be good models for studying human prostatic diseases. The expression of *PCA3* gene was also analyzed in order to predict the exon sites of the assembled sequence and to understand the role of this potential biomarker in the canine prostatic diseases. The absence of amplification of PCA3 transcripts in the studied population leads to the possibility that *PCA3* gene expression might be associated with neoplasic prostatic condition. It is important to continue this research

using new approaches to better characterize this gene and analyze its expression in *Canis familiaris*.

- Concerning the molecular analysis of *IL-6* gene, two *IL-6* gene variants were identified: one located in the coding region (I/5_g105G>A) and another located in the untranslated region of exon 5 (I/5_g440G>A). The results were not indicative of a possible correlation of I/5_g105G>A and I/5_g440G>A variants with susceptibility to develop a specific prostatic disease. However, I/5_g105G>A variant affected the encoded amino acid and may have damage effects for the IL-6 protein and the I/5_g440G>A variant may influence the susceptibility to develop prostatic diseases by affecting the regulatory elements of *IL-6* gene. In this context, further investigations would be of extreme value to clarify the genetic susceptibility profiles of the different canine prostatic disorders.

- The meta-analysis of *IL-6* -174G>C polymorphism revealed that the analysed genetic models were not significantly associated with PCa risk. Nevertheless, concerning the *IL-6* -636G>C polymorphism, the CC and GC genotypes were statistically significantly associated with PCa decreased risk in an Asian population.

In conclusion, the obtained results are an open door to a wide variety of investigations, including the development of new biomarkers and the improvement of therapeutic and preventive approaches for prostatic diseases, with benefits for both human and veterinary medicine.

Chapter VII – References

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ANNEXES

ANNEX I

HOSPITAL VETERINÁRIO DA UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

FICHA DE EXAME ECOGRÁFICO DA PRÓSTATA

Ficha nº. ___ Clínico Responsável: _____ Data: ___/___/_____

IDENTIFICAÇÃO ANIMAL

Nome: _____ Idade: _____ Peso: _____

ID: _____ Cond corporal: _____

Raça: _____

PROPRIETÁRIO Nome: _____ Contactos: _____ / _____

Morada: _____

HISTÓRIA CLÍNICA

Motivo da Consulta: _____

_____

História Médica: _____

_____

_____

_____

EXAME CLÍNICO

Exame de Estado Geral: Temperatura: _____

Freq. cardíaca: _____ Freq. respiratória: _____

Mucosas: _____ Hidratação: _____

Obs: _____

Sintomatologia prostática Ausente ☐

Tenesmo	☐	Perda peso	☐
Hematúria	☐	Dor abdom caudal	☐
Descarga hemorrágica	☐	Disúria	☐
Descarga purulenta	☐	Outros_____	

TOQUE RECTAL

Normal	S ☐	N ☐		
Anormal				
Dimensões	↑ ☐	↓ ☐		
Consistência	N ☐	AN ☐		
Simetria	N ☐	AN ☐		
Estruc flutuantes	S ☐	N ☐		
Sensibilidade	N ☐	AN ☐		

Obs. _____

_____

EXAME ECOGRÁFICO TESTÍCULO

Testículo direito ☐ Testículo esquerdo ☐

Obs. _____

EXAME ECOGRÁFICO ABDOMINAL _____

EXAME ECOGRÁFICO DA PRÓSTATA

Ecogenicidade N ☐ AN ☐ _____

Posição Pelv ☐ Abd ☐ _____

Forma N ☐ AN ☐ _____

Lesões quísticas S ☐ N ☐ Obs Anotar nº _____

Ø e Ecogenicidade
Conteúdo _____

Contornos N ☐ AN ☐ _____

Dimensões

Varrimento Longitudinal

Comprimento _____

Altura _____

Varrimento Transversal

Largura_____

Altura _____

Cálculo do volume da próstata

V= Comprimento × Largura × Altura × 0,523 (Ruel et al., 1998)

Amostras

1- Sangue: centrifugar 2500 rpm 10 min
2- Urina: colhida por sonda após massagem. Colher urina por cistocentese para urinanálise (sedimento e urina tipo II)?
3- Fluido prostático
   Citologia prostática com agulha fina

ANNEX II

HOSPITAL VETERINÁRIO DA UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

AUTORIZAÇÃO PARA COLHEITA DE MATERIAL BIOLÓGICO

1. PROPRIETÁRIO

Nome: _____

Morada: _____

Bilhete de Identidade Nº: _____

2. ANIMAL

Nome: _____ Nº de Identificação: _____

Espécie: _____ Raça: _____ Idade: _____ Sexo: _____

3. INTERVENÇÃO: **Ecografia e massagem prostática. Recolha de sangue, urina, fluido prostático e citologia aspirativa**

4. DECLARAÇÃO

Eu, abaixo assinado, declaro que autorizo o Médico Veterinário _____ a realizar a intervenção descrita, no animal acima referido, actuando de acordo com o seu critério.

Por ser verdade, assino a presente autorização de acordo com o meu documento de identificação.

Vila Real, _____ de _____ de 20___

O Proprietário,

_____

ANNEX III

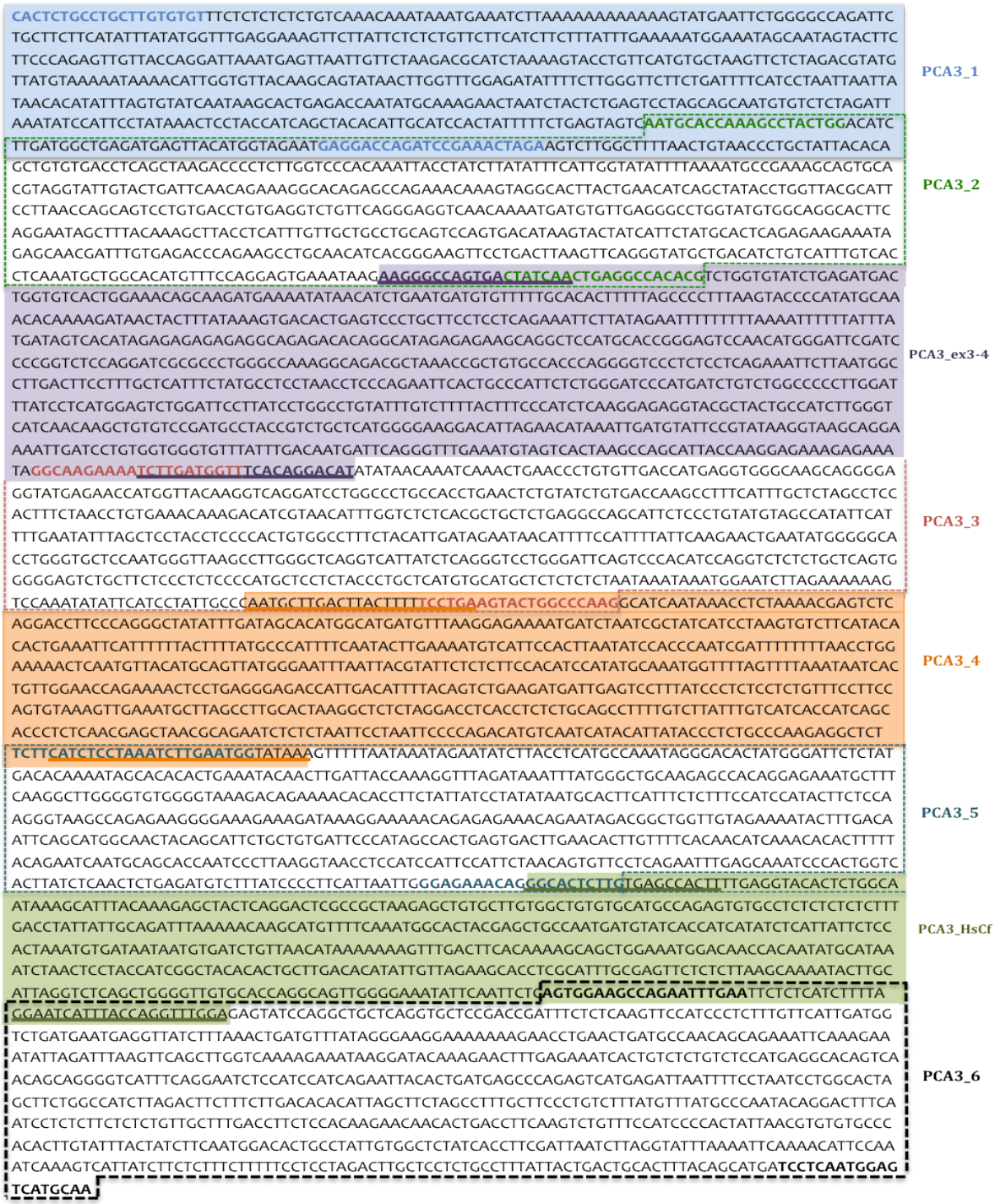
Nucleotide sequences of the *CPSE* gene (GenBank ID: NM_001003284.1) fragments analyzed in the present study. The primers used to amplify the two regions are delimited with grey boxes. In the first fragment are selected the sequences of exon 1 (---) and exon 2 (---). In the second fragment are indicated the sequences of exon 3 (---) and exon 4 (---).

Fragment 1 (416bp)

Fragment 2 (575bp)

ANNEX IV

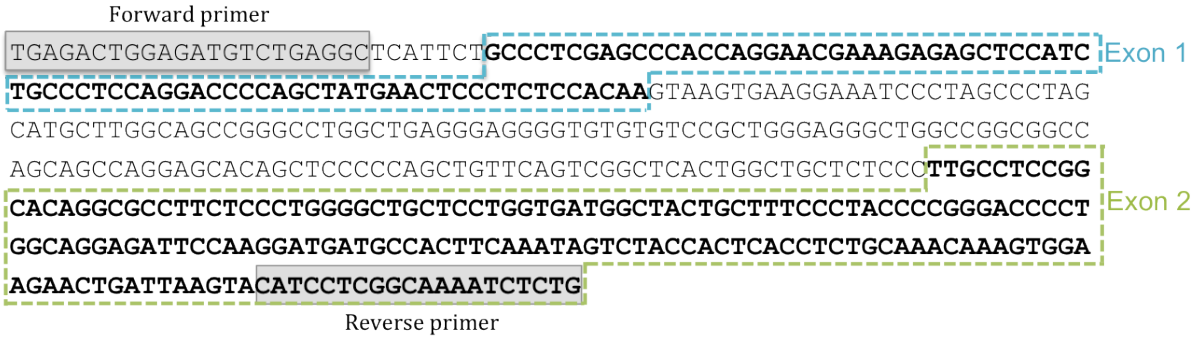
Assembled dog PCA3 gene sequence obtained from the sequencing of PCR products observed in Figure 7. The different sets of primers are emphasized using different colors (PCA3_1, PCA3_2, PCA3_Ex3-4, PCA3_3, PCA3_4, PCA3_5, PCA3_HsCf, PCA3_6).



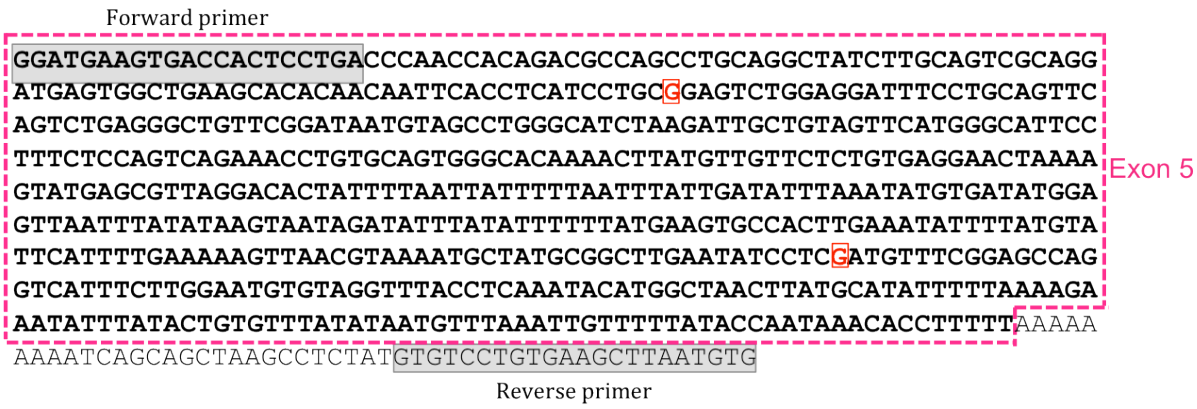
ANNEX V

Nucleotide sequences of the *IL-6* gene (GenBank ID: NM_001003301) fragments analyzed in the present study. The primers used to amplify the two regions are delimited with grey boxes. In the first fragment are selected the sequences of exon 1 (---) and exon 2 (---). In the second fragment is indicated the sequence of exon 5 (---). The positions of the identified genetic variations are evidenced with a red rectangle (□).

Fragment 1 (431bp)



Fragment 2 (630bp)



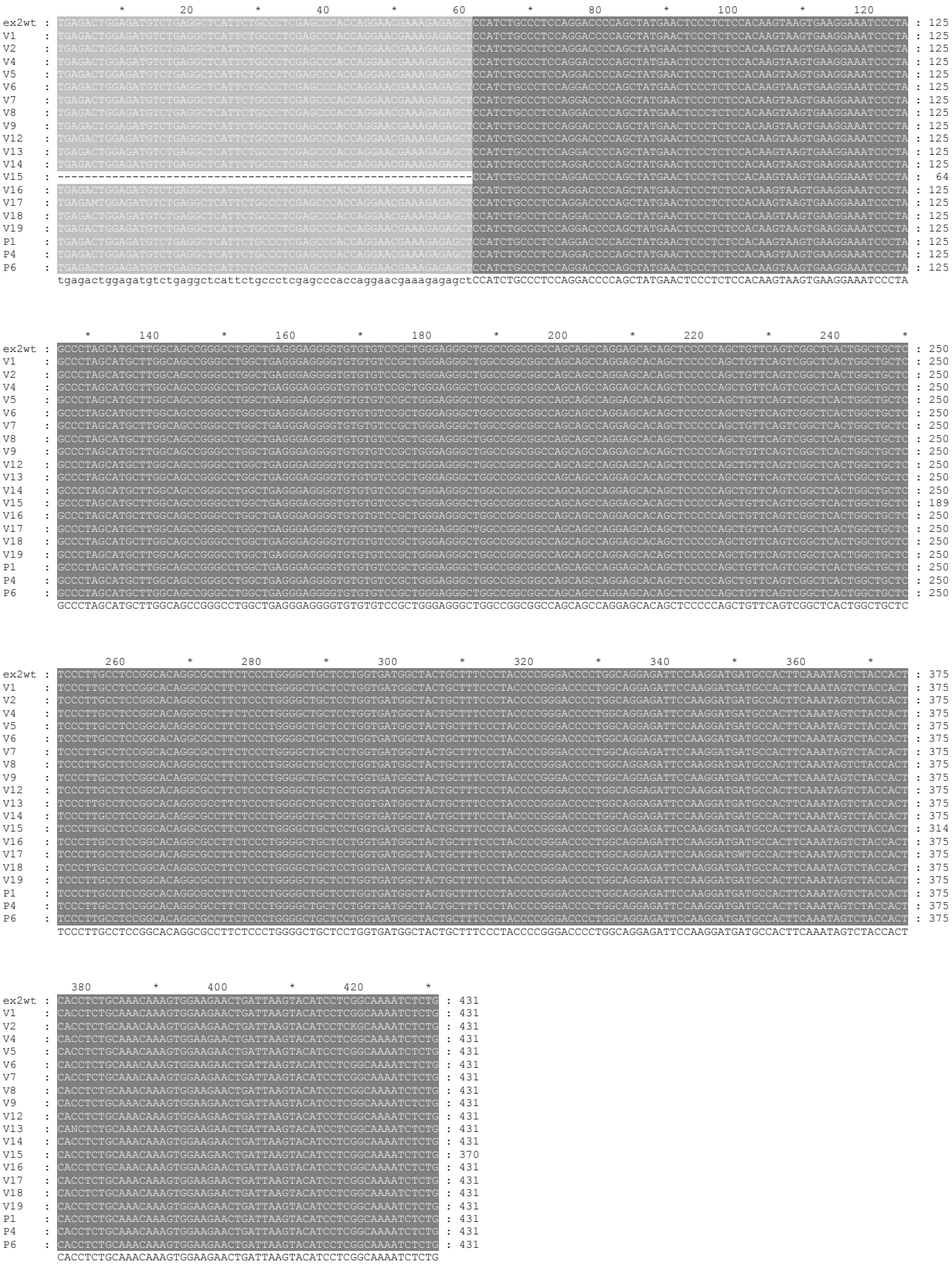
ANNEX VI

Information regarding each set of primers: sequence, melting temperature and length of the amplified fragment.

Gene	Primer set	Sequence	Tm (°C)	Length
GAPDH	GAP_Cf_F	5´- ATGATTCTACCCACGGCAAA -3´	55	176 bp RNA
	GAPDH_Ctrl_R	5´- CTTCTCCATGGTGGTGAAGA -3´		
CPSE	CPSE_Ex1-2_F	5´- CTCCCAGCACTCCTGTCAC -3´	57	416 bp DNA
	CPSE_Ex1-2_R	5´- ACAGTGGGCAGCTGTGAGC -3´		218 bp RNA
	CPSE_Ex3-4_F	5´- GGGTCGCCACAACCTGTC -3´	57	575 bp DNA
	CPSE_Ex3-4_R	5´- TTTTTACCCTCCAAGACACCA -3´		400 bp RNA
IL-6	IL-6_Cf_5UTR_F	5´- TGAGACTGGAGATGTCTGAGGC-3´	57	431 bp DNA
	IL-6_Cf_ex2_R	5´- CAGAGATTTTGCCGAGGATG -3´		
	IL-6_Cf_ex5_F	5´- GGATGAAGTGACCACTCCTGA – 3’	68	630 bp DNA
	IL-6_Cf_3UTR_R	5’ – CACATTAAGCTTCACAGGACAC -3’		
PCA3	PCA3_1_F	5´- CACTCTGCCTGCTTGTGTGT -3´	55	600 bp DNA
	PCA3_1_R	5´- TCTAGTTTCGGATCTGGTCCTC – 3´		
	PCA3_2_F	5´- AATGCACCACCAAAGCCTACTGG – 3´	55	632 bp DNA
	PCA3_2_R	5´- CGTGTGGCCTCAGTTGATAG – 3´		
	PCA3_3_F	5´- GGCAAGAAAATCTTGATGGTT -3´	55	605 bp DNA
	PCA3_3_R	5´- CTTGGGCCAGTACTTCAGGA – 3´		
	PCA3_4_F	5´- AATGCTTGACTTACTTTTTCCTGA - 3´	55	645 bp DNA
	PCA3_4_R	5´- TTTATACCATTCAAGATTTAGGAGATG - 3´		
	PCA3_5_F	5´- TCTTCATCTCCTAAATCTTGAATGG – 3 ´	55	602 bp DNA
	PCA3_5_R	5´- CAAGAGTGCCCTGTTTCTCC – 3´		
	PCA3_6_F	5´- AGTGGAAGCCAGAATTTGAA – 3´	55	772 bp DNA
	PCA3_6_R	5´- TTGCATGACTCCATTGAGGA- 3´		
	PCA3_ex3-4_F	5´- AAGAAGGGCCAGTGACTATCAA – 3´	55	814 bp DNA
	PCA3_ex3-4_R	5´- ATGTCCTGTGAAACCATCAAGA – 3´		
	PCA3_HsCf_F	5´- GGCACTCTTGTGAGCCACTT – 3´	55	511 bp DNA
	PCA3_HsCf_R	5´- TCCAAACCTGGTAAATGATTCC – 3´		

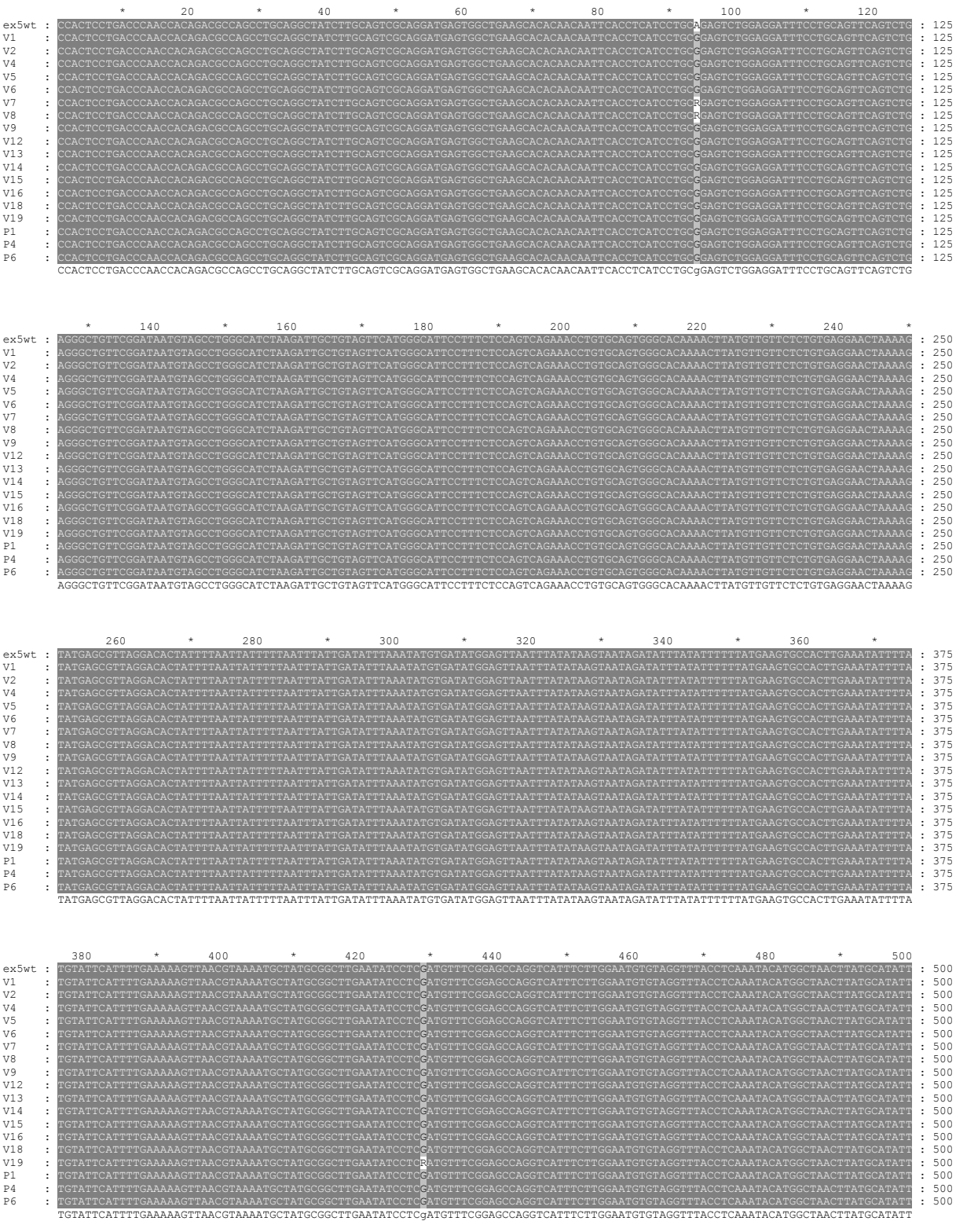
ANNEX VII

Multiple sequence alignment of the selected *IL-6* region (from exon 2 to exon 5).



ANNEX VIII

Multiple sequence alignment of the selected *IL-6* region (from exon 5 to 3'UTR region), allowing the identification of two single nucleotide variations.



```
                 *       520         *       540         *       560         *       580         *       600
ex5wt :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V1    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V2    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V4    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V5    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V6    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V7    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V8    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V9    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V12   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V13   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V14   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V15   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V16   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V18   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
V19   :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
P1    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
P4    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
P6    :  TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA : 609
          TTTAAAAGAAATATTTATACTGTGTTTATATAATGTTTAAATTGTTTTTATACCAATAAACACCTTTTTAAAAAAAAATCAGCAGCTAAGCCTCTATGTGTCCTGTGAA
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