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FACULDADE DE MEDICINA VETERINÁRIA

BOVINE PAPILLOMAVIRUS TYPE 2 INFECTION IN THE PATHOGENESIS OF THE BOVINE ENZOOTIC HEMATURIA-RELATED ONCOLOGICAL PROCESS

JOÃO DE BETTENCOURT BARCELOS COTA

Orientadores: Doutora Maria da Conceição da Cunha e Vasconcelos Peleteiro

Doutora Ana Isabel Simões Pereira Duarte

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Sanidade Animal

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To my wife and my parents

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Abstract

Title: Bovine papillomavirus type 2 infection in the pathogenesis of the Bovine Enzootic Hematuria-related oncological process.

Bovine Enzootic Hematuria (BEH) is a disease that affects cattle and water buffalos in specific regions of the globe, mainly associated with the chronic ingestion of bracken fern (*Pteridium aquilinum)* whose major characteristic is the development of urinary bladder tumors. One of the regions where this disease is endemic is the Azores Archipelago, Portugal. In these Atlantic islands bracken fern finds the appropriate conditions for continuous growth in the farmlands, and thus this toxic plant is frequently available for cattle consumption. The involvement of the Bovine papillomavirus type 2 (BPV2) in the genesis of this disease has been pointed out to be paramount, but some aspects involving the association between bracken fern and Papillomavirus remain unclear. This work aimed to study the role of this infectious agent in the oncological process observed in BEH, and is divided into seven chapters.

In the first chapter, the major features, the implications and the etiology of this disease, with a special focus on ptaquiloside and BPV2, are reviewed. Additionally, urinary bladder tumors in man, and the genetic alterations associated to the disease, are briefly revised.

The second chapter contains the specific objectives that were sought with the present work, and that resulted in the four experimental chapters that follow.

In the third chapter, a detailed histopathological characterization of the urinary bladder lesions found in BEH-affected cattle from the Azores Archipelago which were used in the experimental work, is presented. Furthermore, an immunohistochemical study of the expression of cytokeratins in the epithelial neoplasms is also presented. Our results support previous findings that most urinary bladder BEH-associated lesions are neoplastic, and have epithelial origin. The decrease in the number of neoplastic cells expressing cytokeratin 7 in urinary bladder urothelial tumors was associated with increasing pathological grade and stage, whereas the decrease in the expression of cytokeratin 20 was only associated with increasing stage.

The fourth chapter is dedicated to the prevalence and transcriptional activity of BPV2 in the urinary bladder lesions of BEH-affected cattle from the Azores Archipelago. The results obtained show that, though BPV2 is widely distributed within the bovine

population of the Azores, the viral loads determined are very low, suggesting that BPV2 could be inactive, since no transcriptional activity was detected.

In the fifth chapter a quantitative and qualitative gene expression study is presented, comparing the expression levels of cell cycle controlling genes and of a growth factor receptor gene in BPV2 positive and negative epithelial and endothelial bovine urinary bladder tumors of BEH-affected cattle. The expression of *TP53*, *MDM2* and *CCND1* genes was above normal, but no differences between BPV2 positive and negative epithelial and endothelial tumors were found. The expression of the *EGFR* gene was lower both in BPV2 positive epithelial and endothelial tumors when compared with BPV2 negative ones. This possible association between BPV2 infection and lower *EGFR* should be further investigated in future studies.

The sixth chapter presents the preliminary *in vitro* study through which the effects of BPV2's oncoproteins in a bovine cell line were assessed. Neoplastic transformation was not achieved, but changes in the cellular growth rate and gene expression patterns were observed, suggesting viral oncoprotein activities yet unknown.

The main conclusions of this work are pointed out and discussed in the seventh chapter. The oncological process found BEH and its association with BPV2 are still a matter for further research. The results presented within this thesis provide a better insight into this subject but also open and support new questions worth investigating in future research.

Keywords: Bovine Enzootic Hematuria, Bladder Tumors, BPV2, Gene Expression, Neoplastic Transformation.

Resumo

Título: Infeção por Papilomavírus Bovino tipo 2 na patogenia do processo oncológico associado à Hematúria Enzoótica Bovina.

A Hematúria Enzoótica Bovina (HEB) é uma doença que afeta bovinos e búfalos em regiões específicas do Globo particularmente relacionada com a ingestão do feto comum (*Pteridium aquilinum*), sendo um dos aspetos mais relevantes o desenvolvimento de tumores na bexiga dos animais afetados. Uma dessas regiões onde a HEB é endémica é o Arquipélago dos Açores, Portugal. O feto comum encontra nestas ilhas Atlânticas as condições apropriadas para o crescimento constante nas pastagens, fazendo com que esta planta tóxica esteja facilmente acessível aos animais criados em regime extensivo. A participação do Papilomavírus bovino tipo 2 (BPV2) também tem sido apontada como crucial na génese da HEB. Porém, alguns dos aspetos relacionados com a associação entre o feto comum e o Papilomavírus permanecem por esclarecer. O presente trabalho teve como objetivo estudar o papel deste agente infecioso no processo oncológico associado à HEB, estando dividido em sete capítulos.

O primeiro capítulo integra a introdução geral ao tema estudado, bem como a revisão das principais características da HEB, das suas implicações socioeconómicas e da etiologia da doença, dando atenção especial ao ptaquilósido e ao BPV2. Os tumores de bexiga em humanos, assim como as alterações genéticas associadas a este tipo de neoplasias, são também alvo de revisão no referido capítulo.

No segundo capítulo estão discriminados os objetivos específicos do presente trabalho, que resultaram na conceção de quatro trabalhos experimentais, apresentados nos capítulos subsequentes.

No terceiro capítulo é apresentada a descrição histopatológica detalhada das lesões de bexiga encontradas em animais afetados por HEB no Arquipélago dos Açores, e que serviram de base ao trabalho efetuado nos diferentes estudos realizados. Adicionalmente, é também apresentado um estudo imuno-histoquímico relativo à expressão de proteínas do citoesqueleto em tumores epiteliais da bexiga desses mesmos animais. Todas as amostras de lesões de bexigas de bovinos afetados pela HEB foram colhidas nos matadouros da Ilha de São Miguel e Ilha Terceira durante o ano de 2012. O estudo imuno-histoquímico foi executado recorrendo ao uso de anticorpos comerciais específicos para a deteção das citoqueratinas 7 e 20. Os resultados obtidos confirmaram a grande variedade

histológica de lesões que podem ser identificadas em animais afetados pela HEB. A maioria das lesões identificadas eram neoplásicas, tanto benignas como malignas, excedendo largamente o número das lesões inflamatórias. De entre as lesões neoplásicas, as mais frequentes eram de origem epitelial, destacando-se o carcinoma do urotélio, já que este foi o tipo histológico mais diagnosticado. Foram também identificadas lesões neoplásicas com origem vascular, especificamente hemangiomas e hemangiossarcomas. O estudo imuno-histoquímico revelou que na maioria das lesões neoplásicas epiteliais benignas e malignas de baixo grau de malignidade, grande parte das células apresentavam expressão das citoqueratinas 7 e 20, por vezes com um padrão semelhante ao observado no urotélio normal. A diminuição do número de células neoplásicas que apresentavam expressão destas proteínas do citoesqueleto estava associada com o agravamento do estadio com invasão da túnica muscular e com o aumento do grau de malignidade no caso da citoqueratina 7. A diminuição da expressão da citoqueratina 20, por outro lado, estava associada apenas com o agravamento do estadio de invasão.

O quarto capítulo é dedicado ao estudo da prevalência do BPV2, e à sua possível atividade transcricional, nas lesões da bexiga de bovinos afetados pela HEB do Arquipélago Açoriano. Para tal foi desenvolvido um sistema de PCR quantitativo, baseado na tecnologia TaqMan™, recorrendo à amplificação específica dos genes L1 e E5 do BPV2, bem como à deteção dos seus RNA mensageiros. Papilomas cutâneos de bovinos foram utilizados como controlos positivos. O genoma do BPV2 foi detetado em baixos títulos nas amostras colhidas nos matadouros da Ilha de São Miguel e Ilha Terceira, variando entre 0.00017 a 0.072 cópias por célula. Não foi possível associar a presença do genoma do papilomavírus em estudo com qualquer tipo histológico de lesão de bexiga. Não foi detetado RNA mensageiro viral em qualquer das amostras positivas a BPV2. Os resultados obtidos demonstram que o BPV2 se encontra disseminado na população de bovinos do Arquipélago Açoriano. Porém, a aparente ausência de transcritos virais sugere o vírus se poderá encontrar num estado de inatividade, sendo plausível propor que se tratará de uma infeção abortiva.

O quinto capítulo descreve o estudo de avaliação da expressão dos genes responsáveis pelo controlo do ciclo celular *TP53*, *MDM2* e *CCND1*, bem como do gene do recetor de fator de crescimento *EGFR*, comparando os seus níveis de expressão entre grupos de tumores epiteliais e endoteliais positivos e negativos a BPV2. Esta avaliação foi executada recorrendo à análise relativa de expressão génica por PCR quantitativo, e confirmada pela deteção das proteínas codificadas pelos genes em causa por imuno-

histoquímica. Os genes *TP53, MDM2* e *CCND1* encontravam-se sobrexpressos na análise quantitativa e na imuno-histoquímica tanto nos tumores positivos como negativos a BPV2, independentemente da sua origem histológica. Para estes genes não foi encontrada nenhuma associação entre o seu nível de expressão e a presença ou ausência do BPV2, quer nos tumores epiteliais como nos endoteliais. Por outro lado, o gene *EGFR* apresentou subexpressão na análise quantitativa e na imuno-histoquímica nos grupos de tumores epiteliais e endoteliais BPV2 positivos, quando comparada com o nível de expressão nos tumores BPV2 negativos. A presença ou ausência do BPV2 não parece ter influência na expressão dos genes controladores do ciclo celular, verificando-se que no processo oncológico associado à HEB o nível de expressão destes genes encontra-se acima dos valores normais. É de destacar a associação entre presença de BPV2 e níveis mais baixos de expressão do gene *EGFR*. Esta aparente relação entre a infeção por BPV2 e a diminuição da expressão do gene *EGFR* em tumores da bexiga de bovinos era até agora desconhecida e deverá investigada em trabalhos futuros.

O sexto capítulo contém o estudo preliminar, em condições *in vitro*, no qual os efeitos das oncoproteínas do BPV2 foram testados numa linha celular de rim de bovino. A linha celular MDBK foi infetada utilizando retrovírus, incapazes de se replicarem, contendo as sequências dos genes E5, E6 e E7, que codificam as respetivas oncoproteínas. Foram avaliados os efeitos da expressão das oncoproteínas na morfologia e comportamento celular, bem como no padrão de expressão nos genes celulares acima referidos. A expressão das oncoproteínas virais não induziu nenhuma alteração evidente na morfologia celular. Por outro lado, as células que expressavam as oncoproteínas E5 e E6 apresentaram taxa de divisão celular superior em relação às células que expressavam a oncoproteína E7. Foi também notório que as células que expressavam continuamente a oncoproteína E7 apresentaram uma taxa de divisão celular reduzida quando comparadas com as células controlo. Quando analisados os padrões de expressão dos genes celulares constatou-se que as células contendo o gene e expressando a oncoproteína E7 apresentavam sobrexpressão muito marcada do gene celular *TP53*. Este achado sugere que algumas das funções das oncoproteínas do BPV2 poderão estar ainda por desvendar, sendo necessário mais estudos de modo a clarificar estes resultados.

As conclusões deste trabalho são apresentadas e discutidas detalhadamente no capítulo sete. Não é possível descartar por inteiro a atuação do BPV2 como ator participante no processo oncológico encontrado nos animais afetados pela HEB, embora o seu eventual papel pareça ser negligenciável. Com o presente trabalho foi possível esclarecer algumas

das questões relacionadas com o processo oncológico associado à HEB, embora outras tenham sido levantadas. A associação entre o BPV2 e a HEB mantém-se como um tópico de pesquisa interessante para estudos futuros. Os resultados apresentados nesta tese são o mote para estudos posteriores tanto na área do processo oncológico com sede na bexiga, bem como na área de investigação da transformação neoplásica relacionada com os Papilomavírus.

Nota: As siglas identificativas do papilomavírus bovino e do ácido ribonucleico foram escritas propositadamente na versão em Língua Inglesa por ser a que é mais facilmente reconhecida, sem que o autor com isso queira demonstrar menor respeito pela Língua Portuguesa. Já para a sigla identificativa da Hematúria Enzoótica Bovina foi selecionada a Língua Portuguesa.

Palavras-chave: Hematúria Enzoótica Bovina, Tumores de Bexiga, BPV2, Expressão Génica, Transformação Neoplásica.

Publications

The results presented on this thesis are part of the following scientific publications:

- João Bettencourt Cota, Sandra Carvalho, Ana Duarte, Luís Tavares, Maria da Conceição Peleteiro (2015). Cytokeratins in bovine urothelial bladder tumors: expression and prognostic value. Proceedings of the 1st National Symposium Biomarkers in Animal Science and Veterinary Sciences - An Interdisciplinary Approach, March, Évora, Portugal.

- João B. Cota, Maria C. Peleteiro, Lisa Petti, Luís Tavares, Ana Duarte (2015). Detection and quantification of bovine papillomavirus type 2 in urinary bladders and lymph nodes in cases of Bovine Enzootic Hematuria from the endemic region of Azores. Veterinary Microbiology, Vol 178, Issues 1–2, 9 July 2015, Pages 138–143.

- J. B. Cota, A. Duarte, L. Tavares, M. C. Peleteiro (2015). EGFR expression in BPV2 positive urinary bladder urothelial carcinomas – a preliminary study. Proceedings of the HPV2015, 30th International Papillomavirus Conference & Clinical and Health Workshops, September, Lisbon, Portugal.

Contents

List of Figures

List of Tables

List of Abbreviations

BEH	Bovine Enzootic Hematuria
Bp	Base pairs
BPV	Bovine papillomavirus
BPV1	Bovine papillomavirus type 1
BPV2	Bovine papillomavirus type 2
cDNA	Complementary deoxyribonucleic acid
CK7	Cytokeratin 7
CK20	Cytokeratin 20
DNA	Deoxyribonucleic acid
Hg	Hemangioma
HgS	Hemangiosarcoma
HPV	Human papillomavirus
HPV6	Human papillomavirus type 6
HPV16	Human papillomavirus type 16
kDa	Kilodalton
LCR	Long control region
MDBK	Madin-Darby bovine kidney
MHC I	Major histocompatibility complex class I
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
PDGFR-β	Platelet-derived growth factor receptor beta
PV	Papillomavirus
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
UB	Urinary bladder
UBT	Urinary bladder tumors
UC	Urothelial carcinoma

UP	Urothelial papilloma
WHO	World Health Organization

Chapter I – Introduction and literature review

Introduction

Bovine Enzootic Hematuria (BEH) is a disease with substantial health, and economic, impacts in some regions of the Globe, including the Azores Archipelago, Portugal. Different research groups from the various BEH-affected countries have been on the vanguard of the knowledge concerning this disease. Although the association between cattle grazing in bracken fern infested pastures and the occurrence of BEH had already been perceived some time before, the actual role of bracken in the development of urinary bladder tumors, one of the clinical manifestations of the disease, was only proven in 1969 by Pamukcu and Price. With this finding the real carcinogenic potential of bracken was uncovered, not only for bovine but also for human health, this being the starting point of further studies concerning the carcinogenic components of bracken fern, a very widely distributed plant. The actual impact of bracken in human health is still under investigation. The complexity of BEH was highlighted when the suspicions of the involvement of an infectious agent were confirmed. The same agent responsible for the cutaneous papillomas in cattle, the Bovine Papillomavirus (BPV), was proposed to co-participate in the carcinogenic process taking place in the urinary bladders of cattle (Campo et al., 1992). Since then, many research groups have focused on the interaction between the carcinogenic compounds of bracken and the viral agent, investigating the role of BPV in the development of bladder tumors. This has been a major field of research in the BEH scenario. Some of the more recent reports propose a revolution in the way we look at Papillomaviruses and their biology, and most of all highlight the particularities of BPV.
The similarities between some of the urinary bladder neoplasms found in BEH-affected cattle and in humans, and the interaction of environmental factors and infectious agents turn this natural occurring disease into an interesting and valuable animal model of carcinogenesis (Carvalho et al., 2006; Roperto et al., 2010). Furthermore, the BPV model can be very useful to better understand the mechanisms of Papillomavirus-associated neoplastic transformation.

Therefore, the main objective of this thesis was to evaluate the role of BPV2 on the oncological process found in BEH. The prevalence of this infectious agent was assessed in the Azores Archipelago, a BEH endemic region, based on a large sample of bladders collected in the region using a quantitative PCR (qPCR) method. The possible

involvement of BPV2 in the alterations of the cell cycle control pathways found in urinary bladder tumors of cattle was also studied through the comparison of gene expression levels. Finally, in an *in vitro* model, the actions of the BPV2's oncoproteins were assessed in bovine kidney epithelial cells.

These studies will hopefully contribute for a better understanding of the role of BPV2 in the oncological process in BEH, and also of the mechanisms of BPV-associated cellular transformation.

Literature review

1.1 Bovine Enzootic Hematuria

BEH is an ubiquitous disease that affects cattle and water buffalos in particular geographic areas, prominently associated with continued consumption of the bracken fern (*Pteridium aquilinum*) (Figure 1), whose main clinical feature is hematuria (Dawra and Sharma, 2001).The disease has a chronic evolution being first diagnosed, in most cases, in animals with ages ranging between 4 to 6 years. (Dawra and Sharma, 2001). It is believed that a minimum of 2 years of grazing in bracken infested pastures are necessary for the development of urinary bladder tumors (UBT), the main source of hematuria observed in affected animals (Dawra and Sharma, 2001). If hematuria is the overt clinical sign of BEH, the presence of multiple neoplastic lesions in the urinary bladder is the major macroscopic feature of the disease. Although up to 90% of the cases the hematuria originates from these UBT, animals with non-neoplastic lesions, such as inflammatory or hyperplastic lesions, may also present blood in urine (Carvalho et al., 2006).

This disease was first described by Tophan in 1787 and since then other authors have contributed by reporting cases throughout the years, although with several different designations for the same problem such as: “haematuria essentielle”, “cystite hemorragique”, “hematuria des bovides”, “cistite versucosa”, “red water”, “bovine haematuria”, “enzootic haematuria”, “urocystitis”, “haemorrhagica” or “cystite chronique hemorrage” (reviewed by Dawra and Sharma, 2001).

BEH has been recognized in different countries of Africa, Asia, North and South America, Europe, and Oceania (reviewed by Dawra and Sharma, 2001), reflecting the widespread distribution of bracken fern, one of the five most common higher plants on the planet (Taylor, 1990).

Figure 1 – Cow eating bracken fern on a steep uncultivated area Terceira Island, Azores (original).

1.1.1 BEH in the Azores Archipelago

In Portugal BEH has a significant impact in the Azores Archipelago where this syndrome has been well characterized (Pinto et al., 2000; Pinto et al., 2001; Pinto et al., 2004; Pinto, 2010). Dairy production is the main economic sector of this region. In São Miguel Island, and in other Islands of the Azores Archipelago, the farm land is divided into small fields separated by stonewalls or hedgerows, where cattle graze. The herds are moved from one field to another when the amount of grass is insufficient. Bracken fern is commonly seen growing abundantly along the base of these stonewalls or hedgerows, or in poor pasture land. Due to overgrazing or to poor pasture conditions cattle often have difficulty in finding appealing plant species to graze on and bracken is the most abundant available plant (Pinto et al., 2004).

In São Miguel Island, which has about 54.000 dairy cows, during the period between January 2000 and December 2010 the main cause of all whole carcass condemnations in the local slaughterhouse was the presence of UBT (13.7%) (Pinto et al., 2012). It has been estimated that BEH represents a cost of more than 650.000€ per year to the Azorean Government spent in paying insurances and financial compensations to the farmers.

Furthermore, there are the farmers' costs associated with loss of productivity, veterinary assistance, herbicides, among others (Pinto, 2010).

1.1.2 BEH clinical presentation

The course of the disease can be divided in three different stages: pre-clinical, clinical and terminal stage (reviewed by Dawra and Sharma, 2001).

In the first stage, the animals do not present any clinical signs or systemic disturbances, as just a few erythrocytes are passed in the urine.

The clinical stage is characterized by the transition of the normal color of urine to a vivid red due to the passage of large amounts of intact erythrocytes, which gradually increases with time. The hematuria may disappear for a period of time and reappear weeks or months later (reviewed by Dawra and Sharma, 2001). This intermittence can be observed in animals in the late phase of gestation, a period when hematuria is more frequent, followed by the stoppage of this clinical sign during postpartum and reappearance in the following gestation (Pinto, 2010).

In the terminal stage the animals affected by BEH become emaciated, present a severe anemia and hypoproteinemia resulting in sub-mandibular edema. The passage of large blood clots in their urine may interrupt the urine flow, especially in males as a result of the length and width of the urethra, and may cause secondary complications as cystitis, pyelonephritis, hydronephrosis and ascending bacterial infections, leading ultimately to death (reviewed in Dawra and Sharma, 2001; Pinto, 2010).

1.1.3 BEH and Urinary bladder tumors

As mentioned before, BEH is a chronic disease of cattle and water buffalos with a neoplastic component, and the primary implicated organ of this disease is the urinary bladder. One of the first works regarding the histological nature and characteristics of the lesions present in the urinary bladder of BEH-affected animals in Turkey was published in 1957, by Pamukcu. Later, similar studies from India and Kenya, BEH endemic regions, were also published (Mugera and Nerito, 1968; Nandi, 1969). In 1976 Pamukcu, Pryce and Bryan published the results of the study of 139 naturally occurring and 20 bracken-induced urinary bladder tumors. In this study the size and number of lesions present in

the urinary bladder varied greatly. Of the cases of natural occurring tumors 35% were only epithelial tumors, 54% were mixed epithelial and mesenchymal tumors and 9% of the cases were only mesenchymal tumors (Pamukcu et al., 1976). The histologic classification of the lesions varied, with papillomas, adenomas, transitional cell carcinomas (urothelial carcinomas), squamous cell carcinomas, hemangiomas, hemangioendotheliomas, fibromas, among others (Pamukcu et al., 1976). This work was one of the first to illustrate the heterogeneity present in BEH-associated tumors. Not long after this, another study was published regarding natural cases of BEH in Queensland, describing the presence of hemangiomas, hemangiosarcomas, urothelial carcinomas, papillomas, fibromas and adenomas in the urinary bladders of 19 affected cows (McKenzie, 1978), supporting the reports of lesion heterogeneity described by Pamukcu and colleagues, in 1976. Several years later other groups studying BEH added information with reports of the different histological types of tumors identified in urinary bladder of cattle from China (Xu, 1992), the Black Sea region of Turkey (Özkul and Aydin, 1996), Brazil (Peixoto et al., 2003) and from São Miguel Island, Azores, Portugal (Carvalho et al., 2006). In some studies the number of mesenchymal tumors in pure form exceeded the number of epithelial tumors in pure form (Özkul and Aydin, 1996; Xu, 1992), where in others the very opposite was observed (Table 1) (Carvalho et al., 2006; Pamukcu et al., 1976). Considering the major studies on urinary bladder lesions in BEH the most commonly diagnosed tumors were urothelial papilloma, adenoma, urothelial carcinoma, adenocarcinoma, squamous cell carcinoma, hemangioma, fibroma, hemangioendothelioma and hemangiosarcoma (Carvalho et al., 2006; Pamukcu et al., 1976; Peixoto et al., 2003). The variability between the results of different reports is yet another confirmation of the multiplicity of lesion presentation that can be found in the urinary bladders of the affect animals (Meuten et al., 2004).

In the study of Carvalho and colleagues the histopathological classification papillary neoplasm of apparently low malignant potential (PNALMP) was proposed as this neoplasm shared morphological features of both papillomas and urothelial carcinomas but could not be clearly be diagnosed as neither one of them (Carvalho et al., 2006). This tumor type has its equivalent in the human classification of UBT as papillary urothelial neoplasm of low malignant potential (Eble et al., 2004). The word "apparently" was inserted by the authors as no studies regarding the progression of these tumors could be performed in the cows that had already been slaughtered (Carvalho et al., 2006)

Table 1 – Comparison of percentages of tumor types identified in BEH related studies (Adapted from Carvalho et al., 2006)

Details of tumors	Carvalho et al., 2006	Ozkul and Aydin, 1996	Xu et al., 1989	Pamukcu et al., 1976
Epithelial in pure form	51.2%	35.3%	36.7%	35.2%
Benign	8.3%	24%	NI	17%
Malignant	42.9%	4%	NI	16.5%
Epithelial in combination with mesenchymal	31.4%	25.4%	17.5%	53.9%
Benign	5.1%	19%	NI	9.3%
Malignant	26.3%	4%	NI	44.6%
Mesenchymal in pure form	17.4%	46.6%	45.4%	9.3%
Benign	12.9%	43.2%	NI	5.7%
Malignant	4.6%	3.4%	NI	3.6%
Total number of bladders with tumors	373	815	354	139
Total number of tumors identified	870	1063	NI	NI

NI – not indicated.

In 2010, a report was published where 400 bovine urinary bladder urothelial tumors and tumor like lesions were classified according to the World Health Organization (WHO) criteria for human urothelial tumor classification (Roperto et al., 2010). In this report the authors suggested the adoption of the 2004 WHO classification of human UBT (Eble et al., 2004) for bovine tumor classification, as they share morphological features with the ones found in humans (Roperto et al., 2010). Although the similarities in the growth pattern of urinary bladder epithelial lesions of cattle and man are high, the spectrum of lesion types identified and their frequency can be very different. The most striking difference is the development of endothelial derived tumors, mainly hemangiomas, which can be very frequent in cattle, accounting up to 29.5% of all tumors diagnosed (Carvalho et al., 2006), and that are considered to be rare in humans (Eble et al., 2004). Another example is the occurrence of hemangiosarcomas (named angiosarcomas in human pathology) in the urinary bladders of BEH-affected cattle, very rarely diagnosed in the urinary bladder of humans (Eble et al., 2004).

Additionally, the behavior of the tumors found in the urinary bladders of cattle and humans is apparently diverse. In some studies in bovine, metastases of primary urinary bladder epithelial tumors were detected mainly in the regional lymph nodes, and in smaller number in other organs, ranging from 5% to 15.6% (Carvalho, 2008; Pamukcu et al., 1976; Xu, 1992), some authors reporting that no metastatic lesions were found (Peixoto et al., 2003). In humans about 40% of infiltrative UBT metastasize to the regional lymph nodes (Epstein, 2005). In a recent study the preferential sites of UBT metastasis in humans were the lymph nodes, bones, lungs, liver and the peritoneum (Shinagare et al., 2011). Behavioral characteristics as tumor recurrence or progression are difficult to assess in urinary bladder tumors of BEH-affected cattle as the lesions are mainly detected at slaughter and thus no information can be retrieved regarding the follow up (Carvalho et al., 2006).

The exact mechanisms through which different histologic types of tumors arise from the urinary bladder within the same disease process are not fully understood, but it has been suggested that the amount and the duration of exposure to bracken can have a role in the nature and severity of the lesions (Shahin et al., 1998b).

1.2 Etiology of BEH

Bracken fern consumption has long been associated with BEH as the major causing factor for the development of the disease (Price and Pamukcu, 1968; Hopkins, 1986; Bringuier and Jean-Blain, 1987). In 1959, the discovery that the agent responsible for bovine cutaneous papillomas could also originate UBT (Olson et al., 1959) led to the speculation that this agent could be associated with BEH. The synergic effect of that infectious agent, the BPV, and bracken fern in the development of BEH would be postulated more than 30 years later (Campo et al., 1992). Recently a diet deficient in minerals was proposed as another etiological factor in BEH. These deficiencies could directly affect the cellular mechanisms of prevention/repair of damaged DNA among others, or indirectly, induce a deviant feeding behaviour, or perversion of appetite of cattle (Pinto, 2010).
In sequence, the two globally accepted etiological factors associated with BEH will be described in detail, bracken fern and bovine papillomavirus.

1.2.1 Bracken Fern

Bracken fern, *Pteridium aquilinum* (Figure 2) belongs to the Dennstaedtiaceae family of plants, and it is divided in two subspecies, *P. aquilinum aquilinum* and *P. aquilinum caudatum*, with 12 described varieties between the two subspecies (Tyrion, 1941). It is a plant composed by a rhizome that can reach 2-2.5cm in diameter, from which the stems of the plant arise. Lateral shoots arise from the stem of the plant, producing large fronds and several dormant fronds. The roots which originate from the rhizome can reach a depth as long as 50 cm into the soil (Vetter, 2009).
Bracken fern is one of the most common plants in the world, taking its place in the top five ranking (Taylor, 1990; Smith and Seawright, 1995). The only restrictions on bracken's geographic distribution are lack of humidity and extreme temperatures (Smith and Seawright, 1995). Bracken possesses an extremely high rate of reproduction, the capability for rapid re-growth, a adaption to ecological conditions and an increasing rate of covered surface as main biological features (Vetter, 2009), making it a very skilled invasive plant. In Portugal, bracken is common in the northern region of the mainland and in the Azores and Madeira Archipelagos (reviewed in Gil da Costa, 2011).

A large number of chemical compounds with varied biological activities can be isolated from bracken (Gil da Costa et al., 2012). These compounds cause different chronic and acute problems in different animal species as well in man (Vetter, 2009). Five bracken induced syndromes have been described to occur in domestic animals: thyamine deficiency in horses and sheep, acute hemorrhagic disease or acute bracken poisoning in cattle and sheep, progressive retinal degeneration, also called bright blindness, in sheep, bovine enzootic hematuria in cattle and upper alimentary tract carcinomas in cattle and sheep (Smith, 2004). The latter two syndromes are characterized by the development of neoplasms due to long periods of exposure to bracken toxins.

Figure 2 – Bracken fern. Terceira Island, Azores (original).

1.2.1.1 Bracken-associated carcinogenesis

In the second half of the 20th century bracken was believed to be the cause of hematuria along with the formation of polyps/tumors in the urinary bladders in cattle, when it was consumed for long periods of time (Rosenberger and Heeschen, 1960), thus suggesting that bracken should have a carcinogenic effect. Some years later this suspicion was confirmed in rats, when bracken induced the development of intestinal carcinomas (Evans

and Mason, 1965). This was the first of several studies proving the carcinogenic capabilities of bracken. Not long after bracken was proven to induce UBT in cows (Pamukcu et al., 1967; Price and Pamukcu, 1968) and with these results bracken ingestion was finally proven to be the cause of UBT. The studies with rats continued and the findings of Evans and Mason (1965) were confirmed in another report of UBT having been induced by bracken (Pamukcu and Price, 1969). The effects of bracken were described to be similar to the ones produced by X-radiation and radiomimetic chemicals, as high doses in short periods of time (hours) could cause convulsions and immediate death. On the other hand exposure of the animal to low doses for long periods (years) could lead to the induction of gene mutations and development of tumors (Evans, 1968). Different groups carried on studying the carcinogenic effect of bracken in rodent animal models culminating in the development of different neoplasms in the intestine, urinary bladder and mammary gland in rats (Hirono et al., 1970, 1972, 1983), and leukemia and pulmonary tumors in female Swiss mice (Pamukcu et al., 1972). The capability of bracken to induce the formation of tumors in different organs was undeniable, but the carcinogenic compound was described only in 1983 by two different research groups that named it aquilide A (van der Hoeven et al., 1983) and ptaquiloside (Niwa et al., 1983), which was suggested to be bracken's main carcinogen (Hirono et al., 1984a). Ptaquiloside would be the widely adopted designation for this unstable norsesquiterpene glucoside.

On the course of the search for all of bracken's carcinogenic features other toxic compounds were described as having carcinogenic potential. Shikimic acid (Evans and Osman, 1974) and quercetin (Pamukcu et al., 1980) were pointed out as bracken carcinogens but later studies failed to reproduce the same results and did not support those proposals (Hirono et al., 1981, 1977), leaving ptaquiloside as the leading toxin in bracken-associated carcinogenesis. For this reason only ptaquiloside will be described in detail.

1.2.1.2 Ptaquiloside

Ptaquiloside or ptaquiloside analogues can be found in several ferns other than *Pteridium aquilinum* (Saito et al., 1989), but due to its world dissemination and association with disease bracken remains the foremost important fern regarding animal health (Smith, 1997). Quantification of ptaquiloside in different types of ferns and in the different parts of the plant was performed by multiple research groups from Asia, Europe and South

America (Alonso-Amelot et al., 1992; Pinto et al., 2004; Rasmussen et al., 2008; Saito et al., 1989; Smith et al., 1990). The results are similar between reports with the highest concentrations being found mainly in the young and developing parts of the plant, including the croziers, and the lower concentrations in the rhizomes (reviewed in Yamada et al., 2007). In a study carried out on São Miguel Island, the average values of ptaquiloside found in the young parts of bracken collected from the Island's pastures were 3100 µg/g of dry weight (Pinto et al., 2004). These concentrations are considered very high exceeding by far the 500 µg/g of ptaquiloside limit above which values were considered to be high (Smith et al., 1994).

Depending on the pH, ptaquiloside is converted into different compounds: when in an acidic solution ptaquiloside is converted in pterosin B with the liberation of D-glucose (Figure 3). When present in a weak alkaline solution ptaquiloside is rapidly converted in dienone by elimination of D-glucose. Dienone, also known as activated ptaquiloside, is thought to be the actual carcinogen. It is very unstable in acidic conditions, being immediately converted to pterosin B, but can remain stable for at least one hour in alkaline conditions (reviewed in Yamada et al., 2007). It should be noted that alkaline conditions are required for ptaquiloside activation (van der Hoeven, 1985) and these conditions can be found in the esophagus, in the bladder due to urine pH and in the terminal region of the small intestine (Dawra and Sharma, 2001).

Bracken dienone has been describe as a strong alkylating agent in alkaline conditions binding to DNA (Ojika et al., 1987), forming DNA adducts at the N-3 of adenine and at the N-7 of guanine causing DNA breakage (Ojika et al., 1989). This mode of action of the activated ptaquiloside was confirmed in other trials (Kushida et al., 1994; Smith et al., 1994; Prakash et al., 1996). The sites of DNA cleavage where described with a most preferable sequence being 5'- AAAT (cleavage site underlined) (Kushida et al., 1994). In calves fed bracken, DNA adducts were found in the H-*ras* gene in the codon 61, where the adenine was alkylated, in ileal epithelial cells. It was suggested that this alkylation followed by depurination and error in DNA synthesis was the source of the activation of the proto-oncogene H-*ras* in the ileum of those calves (Prakash et al., 1996). Later, the same group, found double mutations in codons 58 and 59 of the H-*ras* proto-oncogene in the mammary glands of rats subjected to intravenous administration of activated ptaquiloside/dienone (Shahin et al., 1998a), suggesting that H-*ras* activation was an early event in the ptaquiloside associated carcinogenesis process. In a study regarding urinary bladder lesions of cattle grazing in bracken infested pastures H-*ras* immunohistochemical

expression was increased in inflammatory and tumorous lesions, although no mutations were found in the codons 12, 59 or 61 (Sardon et al., 2005). Other studies have also proven the damaging effects of ptaquiloside using mammalian cells (Gil da Costa et al., 2012; Matsuoka et al., 1989; Mori et al., 1985) and bacteria (Matoba et al., 1987).

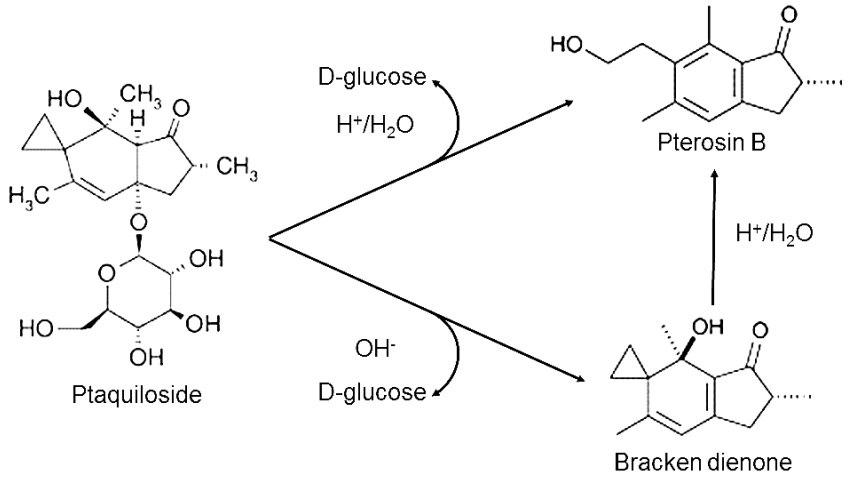

Figure 3 – Reactions of ptaquiloside. Under acid conditions ptaquiloside is converted in to pterosin B, whereas under alkaline conditions is converted into dienone (activated ptaquiloside) (Adapted from Yamada et al., 2007).

1.2.1.3 Bracken as a health hazard

Bracken poses a threat not only for animals, as was discussed previously, but also to man. The possible human exposure routes are diverse (Figure 4) and have been studied by different groups. One of the most implicated routes of human exposure is direct ingestion of the whole or parts of the plant. In some regions of the globe such as Japan and Brazil bracken is seen as food (Alonso-Amelot and Avendaño, 2002; Hirono, 1993; Marliére et al., 1998). The Japanese were the first to conduct studies associating bracken consumption and higher risk of esophageal cancer (Kamon and Hirayama, 1975). A similar work reported higher morbidity and mortality rates due to gastric and esophageal cancer in the Brazilian region of Ouro Preto where the population eats bracken (Marliére et al., 2002).

Another possible route of human exposure is through the ingestion of ptaquiloside contaminated animal products such as milk and meat. The presence of ptaquiloside in

milk of cows fed bracken has been shown to occur (Alonso-Amelot, 1996; Alonso-Amelot et al., 1998), and studies have implicated the consumption of contaminated milk with esophageal and gastric cancer in South America (Alonso-Amelot and Avendaño, 2001; Villalobos-Salazar et al., 1989). Recently, different research groups have focused on the assessment of the presence and amount of ptaquiloside and its risk in animal products, evidencing the presence of ptaquiloside in the plasma, skeletal muscle, liver and milk of cattle under experimental conditions, and in milk of cattle, sheep, goats horse and donkey mares grazing in pasture where bracken grows endemically (Aranha et al., 2014; Fletcher et al., 2011; Francesco et al., 2011; Virgilio et al., 2015). These results highlight the potential health hazard of consumption of animal products from bracken infested areas.

Inhalation of bracken spores has been also been proposed as a risk for human health as in laboratorial conditions this exposure route was associated with cancer and DNA adducts (Povey et al., 1994), but this subject is still unclear.

The possibility of soil and water contamination by ptaquiloside has been studied by a research group from Denmark suggesting that leaching can occur transferring the bracken carcinogen to the soils and underground waters beneath (Rasmussen et al., 2003; Rasmussen et al., 2003; Rasmussen et al., 2005).

As reviewed, bracken can represent a health problem and taking into account the epidemiological studies performed so far it should be faced not only as a livestock hazard but, to some extent, also as a threat to human health.

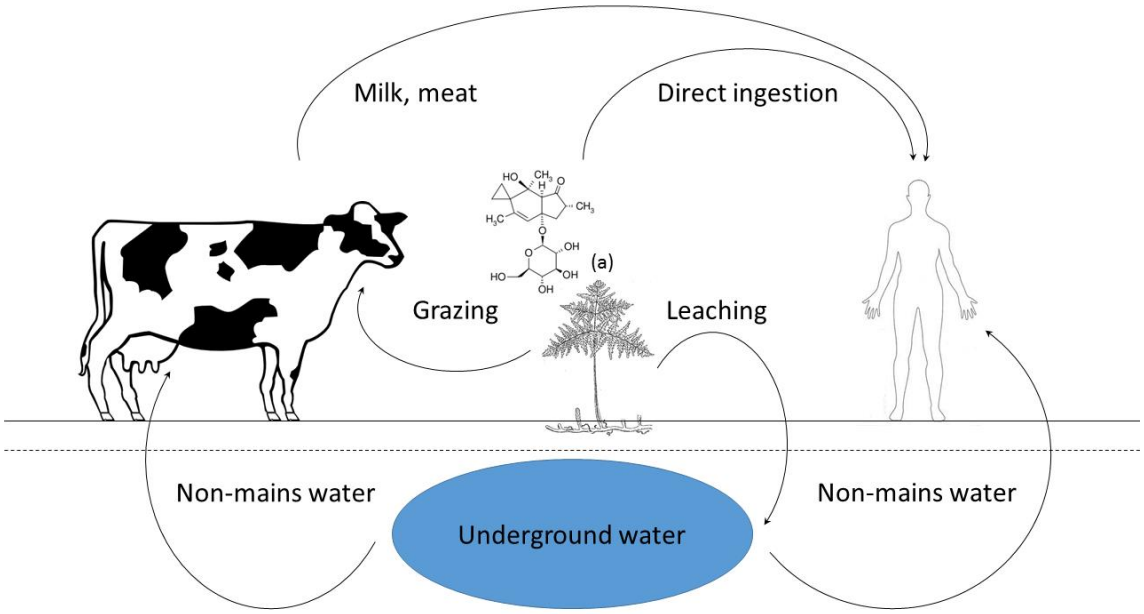

Figure 4 – Possible human ptaquiloside (a) exposure routes (Adapted from Gil da Costa et al., 2012).

1.2.2 Papillomaviruses

Papillomaviruses (PVs) are small, non-enveloped viruses with icosahedral capsids around 50-60 nm in diameter, and with double-stranded DNA genomes that infect the epithelia of vertebrates. They cause the development of exophytic tumors, papillomas, in their natural hosts and in some cases in related species (de Villiers et al., 2004). Their circular genomes have about 8 kb in size and commonly contain 7 to 8 open reading frames (ORFs) (Bernard et al., 2010), divided in to early (E) and late regions (L), and a non-coding region also known as long control region (LCR). Generally, PVs have three oncogenes E5, E6 and E7 that participate in cellular transformation, two regulatory genes E1 and E2 which control transcription and replication, and two late genes L1 and L2 encoding for the capsid proteins (de Villiers et al., 2004). The initial classification of PVs using the classical methods encountered difficulties in the 1970's due to the impracticality to grow these viruses in large scales with the tissue culture methods available at that time, as well as the impossibility to transmit them to laboratory animals (de Villiers, 2013). With the advances of laboratorial techniques the complete genomes of BPV1, Human Papillomavirus (HPV) type 1, HPV6 and HPV16 were reported in the 1980's, and thus began the nucleotide based classification approach (reviewed by de Villiers et al., 2004). The nucleotide sequence of the L1 ORF has been used as the criteria for the classification of PVs, due to its high conservation within the genome (Bernard et al., 2010; de Villiers et al., 2004). Therefore determining the length of the variability in the L1 gene is crucial for PV typing. Differences in more than 10% in the L1 ORF nucleotide sequence from the closest PV type define a new PV type, whereas differences varying between 2 and 10% a subtype or even less than 2%, define a variant (de Villiers et al., 2004). Until now the Papillomaviridae family has 170 HPV types (de Villiers, 2013), and 112 non-human PVs of 54 animal host species, 3 of which affecting birds and 3 affecting reptiles (Rector and Van Ranst, 2013).

1.2.2.1 Bovine papillomaviruses

Bovine papillomaviruses (BPV) are known to infect cutaneous and mucosal epithelia of cattle producing benign lesions (Figure 5). So far, fourteen BPV types have been described and their genomic sequences have been characterized. They belong to four

genera, Deltapapillomavirus (BPV1, 2, and 13), Epsilonpapillomavirus (BPV5 and 8), Xipapillomavirus (BPV3, 4, 6, 10, 11 and 12) and Dyoxipapillomavirus (BPV7) (reviewed by Rector and Van Ranst, 2013). Very recently a novel BPV type has been proposed, BPV14, detected in a feline sarcoid sample which was closely related with the Deltapapillomavirus genera (Table 2) (Munday et al., 2015). From the Amazonia region, Brazil, four new putative BPV types and nine putative BPV subtypes have also been reported to be circulating in the region (da Silva et al., 2015). These recent results suggest that there might be more BPVs than the ones we are aware of, and that it is only a matter of time until new BPV types are reported.

Figure 5 – Cow exhibiting multiple cutaneous papillomas ranging from few millimeters to several centimeters in diameter (original).

The biology of BPVs is very interesting since some of them can behave quite differently from other PVs. Even though PVs are highly species specific some BPV types can cross the species barrier. BPV1 and BPV2 are able to infect equids giving rise to dermal fibroblastic invasive tumors called sarcoids (Nasir and Campo, 2008). Recently BPV13 was also reported to be associated with equine sarcoids (Lunardi et al., 2013b). BPV1 and/or 2 were even detected in cutaneous lesions of different species as zebras, giraffes and tapirs (Kidney and Berrocal, 2008; Löhr et al., 2005; van Dyk et al., 2011). In water

buffalos and yaks BPV1 and/or 2 were found to be associated with cutaneous warts and urinary bladder tumors (Bam et al., 2013; Maiolino et al., 2013; Pangty et al., 2010; Roperto et al., 2013; Silvestre et al., 2009). In a study carried out in India, upper gastrointestinal warts of buffalos were reported to be associated with BPV1, 2 and 5 (Kumar et al., 2015). Another clear example of the ability to cross the species barrier is the newly proposed BPV14 which was found in a feline cutaneous lesion (Munday et al., 2015).

Table 2 – Genomic organization of Bovine Papillomaviruses (Adapted from Rector and Ranst, 2013)

Abbreviation	Classification	Isolated from	Reference
BPV1	Deltapapillomavirus 4	Cutaneous fibropapillomas	Chen et al. (1982)
BPV2	Deltapapillomavirus 4	Cutaneous fibropapillomas	Groff and Lancaster (unpublished)
BPV3	Xipapillomavirus 1	Cutaneous papilloma	Terai et al. (2002)
BPV4	Xipapillomavirus 1	Oral/esophageal papilloma	Patel et al. (1987)
BPV5	Epsilonpapillomavirus 1	Udder fibropapilloma	Terai et al. (2002)
BPV6	Xipapillomavirus 1	Udder papilloma	Jarrett et al. (1984)
BPV7	Dyoxipapillomavirus 1*	Teat papilloma and healthy skin	Ogawa et al. (2007)
BPV8	Epsilonpapillomavirus 1	Cutaneous papilloma	Tomita et al. (2007)
BPV9	Xipapillomavirus 1	Teat papilloma	Hatama et al. (2008)
BPV10	Xipapillomavirus 1	Teat papilloma	Hatama et al. (2008)
BPV11	Xipapillomavirus 1	Cutaneous papilloma	Hatama et al. (2011)
BPV12	Xipapillomavirus 2*	Tongue epithelial papilloma	Zhu et al. (2012)
BPV13	Deltapapillomavirus 4*	Ear cutaneous papilloma	Lunardi et al. (2013)
BPV14	Deltapapillomavirus 4*	Feline sarcoid	Munday et al. (2015)

* Classification not yet approved by the International Committee on Taxonomy of Viruses

It has also been proposed that BPV2 can infect and replicate in more than one type of cell, such as the urothelium (Roperto et al., 2013), the specific epithelium that covers the urinary bladder, the chorionic epithelium of the placenta (Roperto et al., 2012) and peripheral mononuclear blood cells (Roperto et al., 2011).

As mentioned above, BPVs under normal conditions induce the development of benign lesions in their natural host which regress after some time. However, in the presence of environmental co-factors those lesions can progress to form malignant tumors in the urinary bladder and in the upper alimentary canal in cattle (Campo, 2006), hence associating BPV with cancer in cattle.

1.2.2.2 BPV, viral oncoproteins and urinary bladder tumors

Urinary bladder tumors as well as upper gastrointestinal tumors have been proposed to be a result of the interaction between BPV, carcinogens and immunosuppressant toxins present in bracken (Campo, 1997). This scenario where virus and environmental factors form a synergy is, to some extent, the result of the large amount of studies conducted in the framework of bracken fern induced carcinogenesis.

The first suspicion of the involvement of BPV in the development of urinary bladder neoplasms was due to the possibility of inducing urinary bladder tumors with the inoculation of a skin wart suspension (Olson et al., 1959). Some years later the same research group was able to produce not only fibropapillomas in the skin, but also fibropapillomas of the vaginal wall and fibromas in the urinary bladders of calves when injected with suspensions of spontaneous urinary bladder tumors (Olson et al., 1965). These results suggested that the infectious agent could be a causative factor in the genesis of BEH or, alternatively, that it was just a "passenger virus" (Olson et al., 1965). The idea that the infectious agent was a causative factor in the development of urinary bladder tumors was finally demonstrated in 1992 as a result of a study using bracken fed cows. On that study urinary bladder tumors were induced by bracken feeding and 69% of the urinary bladder neoplasms harbored BPV2 DNA (Campo et al., 1992). When those authors searched for BPV2 DNA in naturally occurring bladder tumors they found that 46% of them were positive for viral DNA and that 10% of the non-neoplastic bladder samples were positive. Those results were seen as clear evidence of the BPV participation in BEH. After this report several studies have found BPV1 and 2 associated with cattle urinary bladder cancer in different regions of the globe such as the Azores archipelago,

Brazil, Italy, India and Romania (Balcos et al., 2008; Borzacchiello et al., 2003; Pathania et al., 2012; Resendes et al., 2011; Wosiacki et al., 2005).

As mentioned before, PV-associated cellular transformation is related to the E5, E6 and E7 oncoproteins. The actions of these oncoproteins have been mainly studied in *in vitro* conditions rather than in naturally occurring tumors. E5 is considered to be the major trasforming protein of BPV, and the BPV1 E5 has been the most widely studied BPV oncoprotein (DiMaio and Petti, 2013). This 44 amino acid long protein induces cell transformation through the physical interaction with the platelet derived growth factor β receptor (PDGFR-β) activating it in a ligand independent manner inducing mitogenic stimuli (Drummond-Barbosa et al., 1995; Petti et al., 1991). The nearly identical BPV2 E5 has been been proposed to have the same mechanism of action as it was found to activate the PDGFR-β in naturally ocurring urinary bladder tumors of cattle (Borzacchiello et al., 2006). BPV2 E5 expression in urinary bladder neoplasms of cattle and water buffalos has been reported in different studies (Balcos et al., 2008; Borzacchiello, 2003; Borzacchiello et al., 2009, 2007; Maiolino et al., 2013; Resendes et al., 2011; Roperto et al., 2014, 2008). BPV2 E5 was also reported to bind to the subunit D of the V1-ATPase proton pump in BEH affected cattle urinary bladder tumors (Roperto et al., 2014). The interaction of BPV1 E5 with the components of V-ATPase had been previously described in *in vitro* conditions (Goldstein et al., 1992, 1991). In BPV2 positive urothelial tumors expressing E5 calpain 3 was found to be expressed in an active form, so the involvment of this protein in urothelial carcinomas was suggested (Sante Roperto et al., 2010). The E5 proteins encoded by BPV1 and BPV4 also induce the downregulation of the major histocompatibility complex class I (MHC I) promoting immune evasion of the infected cells (Ashrafi et al., 2002), though this event was never described in naturally occuring tumors despite the similarities of BPV1 and BPV2 E5 proteins.

The E6 oncoprotein of BPV2 has not been the subject of many studies and the best understood E6 is the one encoded by BPV1. These two 137 amino acid long proteins are similar as they contain two zinc-binding domains separated by 36 amino acids (Tomita et al., 2007). The role of E6 in naturally occuring urinary bladder tumors has not been elucidated, but *in vitro* studies have revealed some of the possible actions of BPV1 E6. It has been reported that it can transform cells, not through the degradation of p53 as HPV, but by the downregulation of p53 tumor supressor protein due to the interaction and inhibition of CBP/p300 required by p53 (Zimmermann et al., 2000). BPV1 E6 is also

known to interact with paxilin, a focal adhesion protein disrupting the actin cytoskeleton by blocking the paxilin-vinculin interaction and the focal adhesion kinase (Tong and Howley, 1997; Tong et al., 1997). Later, the same group reported that BPV1 E6 could also interact with AP-1, the trans-Golgi network-specific clathrin adaptor complex, suggesting that E6 could have an effect on the cellular processes which involved the clathrin-mediated trafficking pathway (Tong et al., 1998). In recent studies BPV1 E6 was shown to repress the NOTCH transduction by binding with the mastermind-like protein 1 (MAML 1) and thus delaying keratinocyte differentiation (Brimer et al., 2012). Whether these actions described for E6 do occur in cattle urinary bladder tumors is still uncertain. The role of BPV2 E7 oncoprotein in the development of urinary bladder tumors of catlle is not fully understood, although the expression of this oncoprotein has already been detected in eptihelial and vascular urinary bladder neoplasms (Borzacchiello et al., 2009, 2007). Both BPV1 and BPV2 127 amino acid long E7 proteins do not have the retinoblastoma tumor suppressor (pRB) binding motif present in E7s from other BPVs and HPV (Tomita et al., 2007). The pRB-binding domain is implicated in the E7 mechanism of cellular transformation (Dick and Dyson, 2002). It was proposed that BPV1 E7 does not have the capability of cellular transformation by itself, but instead cooperates with E5 and E6, increasing the transformation capacities of those oncoproteins (Bohl et al., 2001). In spite of this apparent inability to induce cellular transformation, E7 was described to interact with p600, a protein involved in membrane morphogenesis and cell survival (DeMasi et al., 2005) and to inhibit anoikis, due partly to the E7-p600 association (DeMasi et al., 2007).

There are several possible roles of each oncoprotein in urinary bladder tumor development as the many *in vitro* studies point out, but further *in vivo* studies are necessary to clear this issue. In fact, additional studies are required to better understand the actual role of BPV in BEH because many questions related to this matter are yet to be answered.

1.3 Urinary bladder tumors in man

Bladder cancer is one of the most common cancers diagnosed worldwide (Eble et al., 2004), with 74,690 estimated new cases in the USA, in 2014 (Siegel et al., 2014). The rate of bladder cancer in males is three to four times higher than in females, and several risk factors, as tobacco smoke, aromatic amines in paints, caffeine, urologic pathologies among others, have been implicated in the urinary bladder carcinogenesis (reviewed in Mitra and Cote, 2009). A possible role of HPV in the development of urothelial tumors has been suggested by some authors since several HPV types were detected in urinary bladder tumors (LaRue et al., 1995; Shaker et al., 2013; Shigehara et al., 2011) In developed countries urothelial carcinoma is the most commonly diagnosed UBT (Eble et al., 2004). The urothelial tumors are classified according to their morphology, growth pattern and histological features (Table 3) (Eble et al., 2004; Grignon, 2009).

Table 3 – Classification of urothelial neoplasms according to the 2004 WHO/1998 ISUP guidelines (Adapted from Grignon, 2009).

Normal
Hyperplasias
Flat lesions with atypia
Reactive (inflammatory) atypia
Atypia of unknown significance
Dysplasia (low-grade intra-urothelial neoplasia)
Carcinoma *in situ* (high-grade intra-epithelial neoplasia)
Papillary neoplasms
Papilloma
Inverted papilloma
Papillary urothelial neoplasm of low malignant potential
Papillary carcinoma, low grade
Papillary carcinoma, high grade
Invasive neoplasms

The stage of a tumor is evaluated not only by assessing the deepness of urinary bladder tissue invasion but also by the presence of neoplastic cells in the regional lymph nodes and in other organs, according to the TNM system (Table 4) (Sobin et al., 1997). In this system T represents the stage of tumor invasion of the urinary bladder wall, N the presence and the dimension of the metastasis in the regional lymph nodes, if there are any, and M the existence, or not, of distant metastasis.

Table 4 – TNM classification of urinary bladder tumors (Adapted from Sobin et al., 1997)

T – Primary Tumor
TX – Primary tumor cannot be assessed
T0 – No evidence of primary tumor
Ta – Noninvasive papillary carcinoma
TIS – Carcinoma *in situ* (flat tumor)
T1 – Tumor invades subepithelial connective tissue
T2 – Tumor invades muscle
  T2a – Tumor invades superficial muscle (inner half)
  T2b – Tumor invades deep muscle (outer half)
T3 – Tumor invades perivesical tissue
  T3a – Microscopically
  T3b – Macroscopically (tumor can be observed from outside of the bladder)
T4 – Tumor invades any of the following: prostate, uterus, vagina, pelvic wall or abdominal wall
  T4a – Tumor invades prostate, uterus, vagina
  T4b – Tumor invades the pelvic wall, abdominal wall

N – Regional lymph nodes
NX –Regional lymph nodes cannot be assessed
N0 – No regional lymph node metastasis
N1 –Metastasis in a single lymph node 2 cm or less in greatest dimension
N2 – Metastasis in a single lymph node more than 2 cm but no more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
N3 – Metastasis in a lymph node more than 5 cm in greatest dimension

M – Distant metastasis
MX – Distant metastasis cannot be assessed
M0 – No distant metastasis
M1 – Distant metastasis

Stage 0a – Ta, N0, M0
Stage 0is – Tis, N0, M0
Stage I – T1, N0, M0
Stage II – T2a, N0, M0; T2b, N0, M0
Stage III – T3a, N0, M0; T3b, N0, M0; T4a, N0, M0
Stage IV – T4b, N0, M0; Any T, N1, M0; Any T, N2, M0; Any T, N3, M0; Any T, Any N, M1

About 70% of all newly diagnosed cases of urothelial carcinomas are superficial tumors (stages Ta, T1 or Tis), and following surgical resection recurrence occurs in 50-70% of the cases (Kaufman et al., 2009; Knowles, 2006). Although with high recurrence rates, the patient's prognosis is good, since only 10-20% progress to a muscle-invasive lesion. Contrarily, the non-superficial urothelial tumors that are muscle-invasive at the moment of diagnosis have a poor prognosis, with a survival rate of less than 50% at the end of five years (Knowles, 2006). Thus, in human urinary bladders two different tumor presentations with very distinct behaviors are commonly seen: superficial low grade

tumors and muscle invasive tumors. These observations raised the hypothesis that there are two different pathways for urothelial tumor development. The currently accepted theory states that papillary non-invasive tumors arise from urothelial hyperplasia with minimal atypia and that muscle-invasive tumors arise from flat urothelial dysplasia, from which a carcinoma *in situ* is formed and progresses onto a solid invasive tumor (Knowles, 2008). These two different pathways are believed to be the result of different genetic alterations (Figure 6). Low grade papillary tumors harbor at a high frequency deletions on the chromosome 9 and low frequencies of *TP53* gene mutations. The very opposite is seen in carcinomas *in situ*, where *TP53* gene mutations are often detected and low numbers of chromosome 9 alterations are identified (Baffa et al., 2006). Several other chromosome alterations as deletions, gains and amplifications in numerous chromosomes have been identified, at different frequencies of incidence, in non-invasive and invasive bladder tumors (revised by Knowles, 2008). A third pathway has been suggested to exist since there are lesions (T1 papillary carcinomas) that do not fall in the two categories already recognized, and which could arise from flat urothelial dysplasia leading to high-grade papillary carcinomas (Goebell and Knowles, 2010). As mentioned previously, vascular urinary bladder tumors are rarely diagnosed. In fact, non-urothelial tumors account for less than 5% of all of the primary urinary bladder neoplasms in Man (Dahm and Gschwend, 2003). Both hemangiomas and hemangiosarcomas have been identified but are mentioned as rare (Eble et al., 2004), especially hemangiosarcomas as only 13 cases have been reported since the beginning of the 20st century, and that the estimated annual number of new UBT cases worldwide is 260.000 (Bahouth et al., 2015; Eble et al., 2004). Around 10% of all benign soft tissue tumors are hemangiomas (Fletcher et al., 2002), and only 2% of all soft tissue malignant ones are hemangiosarcomas (Coindre et al., 2001). Due to their low relevance in the urinary bladder cancer scenario, only the molecular changes regarding the urothelial tumors will be reviewed.

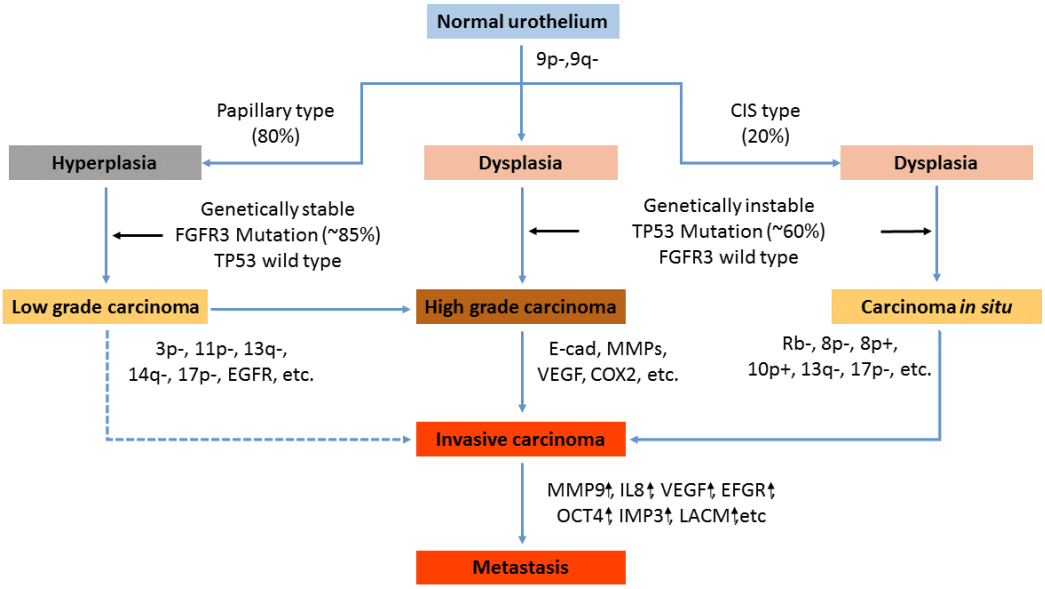

Figure 6 – Two pathways of carcinogenesis are proposed to be on the basis of urothelial tumors (Adapted from Cheng et al., 2011).

Deletions on the chromosome 9 are thought to be an initial event in urothelial carcinogenesis. It is proposed that low grade carcinomas arise from epithelial hyperplasia whereas carcinomas *in situ* and high grade carcinomas arise from epithelial dysplasia. The major differences in these two routes are in the *TP53* and *FGFR3* genes. *TP53* mutations, common in *in situ* and high grade carcinomas, render the cells genetically instable; on the other hand *FGFR3* mutations, frequent in low grade carcinomas, do not alter the genetic stability. Nevertheless, some low grade carcinomas are known to progress high grade carcinomas (dashed line). Several genetic alterations have been associated with the progression of the lesions to invasive carcinomas and to metastatic behavior.

1.3.1 Tumor suppressor genes, proto-oncogenes and their products in urothelial tumors

As any other type of neoplasm, and as was mentioned above, urinary bladder neoplasms arise due to the accumulation of changes in the DNA of somatic cells. These changes can range from a single base pair modification to the loss or gain of a chromosome segment, can be caused by exogenous or endogenous mutagens or can even occur during the reconstruction of damaged DNA (Little, 2010). Some alterations either activate genes that control the signaling pathways that induce cell proliferation, the cellular oncogenes, or inactivate the genes that code for proteins that act as checkpoints of cell proliferation or death, the tumor suppressor genes (Bertram, 2000). Modifications in the tumor suppressor gene *TP53*, in the proto-oncogenes *MDM2* and *CCND1* or in the epidermal growth factor receptor gene (*EGFR*) are amongst the many genes alterations found in several cases of UBT (revised in Baffa et al., 2006).

Without the regulatory mechanisms, cells are susceptible to accumulate further genetic alterations, which can lead to uncontrolled cell proliferation. During this phase the affected cells can acquire the phenotypical traits that ultimately favor the growth of neoplastic cells rather than that of normal cells. These phenotypical traits that define the behavior of neoplastic cells are self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, apoptosis evasion, deregulation of cellular energetics and immune destruction/evasion. Two enabling characteristics are necessary for the acquisition of the referred to phenotypical traits, tumor-promoting inflammation and genome instability and mutation (Hanahan and Weinberg, 2011). All of these characteristics are crucial for neoplastic cell survival and dissemination.

1.3.1.1 *TP53*

The *TP53* tumor suppressor gene, located in the chromosome 17p13, encodes for the p53 protein. This 53 kDa nuclear phosphoprotein is implicated in the control of cell growth and in the maintenance of the genomic stability, acting as a gatekeeper at the G1-S checkpoint of the cell cycle (Levine, 1997). DNA damage, hypoxia and oncogenic stress lead to the activation of p53, driving the affected cells to cell cycle arrest or to apoptosis if the damage is not reparable (Levine, 1997). Alterations in the *TP53* gene are paramount

in the carcinogenic process of several tumors, including the urothelial carcinomas (Cheng et al., 2011). Generally, in human urothelial carcinomas one allele of the *TP53* gene is mutated, thus non-functional, while the other allele is deleted (Ahmad et al., 2012). The majority of mutations found in the *TP53* gene are missense, followed by the non-sense and the silent mutations, respectively (Luis et al., 2007). Under normal conditions p53 has a short half-life. These mutations can result in p53 proteins with an extended half-life due to its resistance to ubiquitin-mediated degradation, leading to nuclear accumulation of a mutant protein which can be detected by immunohistochemistry (Mitra and Cote, 2009). Nuclear accumulation of p53 has been associated with progression of superficial to muscle-invasive urothelial tumors (Esrig et al., 1993) and with decreased overall survival (Esrig et al., 1994). Overexpression of p53 in muscle-invasive urothelial carcinomas was proposed to be an indicator of time to recurrence (Chatterjee et al., 2004) and of clinical outcome of patients undergoing radical cystectomy (Shariat et al., 2004). Although changes in p53 expression were statistically associated with bladder cancer recurrence, progression and mortality (Malats et al., 2005) the interpretation of p53 nuclear overexpression can become very complex, since some *TP53* gene mutations do not lead to increased half-life and other mutations can lead to acquisition of additional functions (Goebell and Knowles, 2010). The relevance of evaluating not only the protein but also the *TP53* gene status was pointed out, since patients with both gene mutation and altered p53 protein had the worst prognosis, but also because some gene mutations apparently did not affect the clinical outcome (George et al., 2007). Furthermore, the site where the mutations occurs in the *TP53* gene may also be an important prognostic factor (George et al., 2007). Studies evaluating *TP53* mRNA expression have also been performed in urothelial tumors. The levels of *TP53* mRNA were found to be increased in the tumor lesions when compared with the normal bladder (Zaravinos et al., 2011). In another study the levels of *TP53* gene expression showed a tendency to discriminate the groups of patients with different clinical outcome (no recurrence, recurrence without progression and recurrence with progression) of non-invasive papillary urothelial tumors (Birkhahn et al., 2010).

The role of p53 in urothelial tumors has been widely studied by several groups during the past years. The assessment of *TP53* mutations by immunohistochemistry, available in many pathology laboratories, is the most commonly used method. Differences in the protocols, antibodies and cutoff values can influence the results obtained for this important biomarker, leading to controversial discussions about its association with tumor

recurrence and patient survival amongst different research centers (Goebell and Knowles, 2010).

1.3.1.2 *MDM2*

The *MDM2* proto-oncogene is located in the chromosome 12q14.3-q15 and encodes a protein which binds and inhibits p53-mediated transactivation (Momand et al., 1992). The MDM2 protein expression is induced by p53, and functions as an E3 ubiquitin ligase which is responsible for the ubiquitination and degradation of the p53 tumor suppressor protein (Honda et al., 1997). Thus, the MDM2-p53 interaction is organized as a negative feedback loop, with MDM2 closely regulating the p53 function (Iwakuma and Lozano, 2003). High levels of p53 induce the transcription of the *MDM2* gene, and the high levels of MDM2 protein leading to lower levels of p53 after proteasomal degradation and in turn, to a reduction of MDM2 protein levels.

The first study on MDM2 in urothelial tumors reported an association of protein overexpression with low grade and low stage tumors, suggesting that this proto-oncogene could be involved in tumorigenesis or in the progression of urothelial tumors (Lianes et al., 1994). Later, in another study, MDM2 protein overexpression in urothelial tumors was suggested to be a result of gene amplification (Barbareschi et al., 1995). Some authors have reported that MDM2 protein overexpression was not an independent predictor of prognostic urothelial tumors (Schmitz-Dräger et al., 1997; Shiina et al., 1999; Yurakh et al., 2006). In spite of this, *MDM2* was identified as a major gene amplification target in urothelial tumors (Simon et al., 2002), and gene amplifications and/or overexpression were found in both superficial and invasive urothelial carcinomas (Goebell and Knowles, 2010; Lindgren et al., 2012; Weilandt et al., 2014). This imbalance in the MDM2-p53 loop, where MDM2 levels are above normal, can disturb the regular control of the cell cycle. Due to high number of gene amplifications, and its impact in the control the cell's regulatory mechanisms, *MDM2* is looked upon as a potential therapeutic target (Chekaluk et al., 2013).

1.3.1.3 *CCND1*

The *CCND1* proto-oncogene is located in the 11q13 chromosome and encodes the protein Cyclin D1, which belongs to a group of proteins, the cyclins, that control the cell-cycle progression and cell differentiation through the activation of the cyclin-dependent-kinases (CDK) (Kopparapu et al., 2013). The passage through the G1-S phase restriction point of the cell cycle is mainly controlled by the interaction of D-type Cyclins with CDK4 and/or CDK6. The complex Cyclin D1/CDK4 promotes the phosphorylation of the pRB, canceling the anti-growth effects of this protein (reviewed by Knudsen et al., 2006;). Thus the complex Cyclin D1/CDK4 can promote the progression of the cell-cycle even without mitogenic stimuli. Cyclin D1 overexpression is proposed to be caused by gene amplification or through chromosomal rearrangements (Baffa et al., 2006). Because *CCND1* gene amplifications/overexpression was found only in 10-20% of all urothelial tumors, the overexpression of Cyclin D1 was proposed to be caused by other pathways. Activation of the mitogen-activated protein kinase (MAPK), PI3-kinase pathways (Knowles, 2006), and mutation in the β-catenin gene (Shiina et al., 2002) were also suggested to be in the origin of Cyclin D1 overexpression in urothelial tumors. During the last years, studies on the significance of Cyclin D1 overexpression in urothelial tumors and patient prognosis have produced dissimilar conclusions. Some studies have reported an association between Cyclin D1 overexpression and poor prognosis (Lopez-Beltran et al., 2004; Yurakh et al., 2006) and others reported the exact opposite (Ren et al., 2014; Sgambato et al., 2002; Tut et al., 2001). In recent studies, higher *CCND1* mRNA expression levels were associated with urothelial tumors when compared with normal urinary bladder tissue, and Cyclin D1 protein expression was associated with lymph node metastasis (Kopparapu et al., 2013). One year later, the *CCND1* gene amplification status and Cyclin D1 protein expression were reported to be independent risk factors of metastasizing bladder cancer (Seiler et l., 2014). The opposing results from different research groups drive the ongoing research on the prognostic utility of the *CCND1*/Cyclin D1 in urothelial tumors, as well as its potentialities as a therapeutic target, due to its role in the cell-cycle control.

1.3.1.4 *EGFR*

The *EGFR,* or *ErbB1*, proto-oncogene is located in the chromosome 7q11.2-q12, and its gene product EGFR belongs to the ErbB tyrosine kinase receptor family with four different members. The extracellular domain of this transmembrane receptor binds to the epidermal growth factor (EGF) and also binds to other ligands (Weintraub et al., 2013). When this receptor is activated by its ligand, homo-dimerization (with another EGFR molecule) or hetero-dimerization (with another receptor of the ErbB family) occurs, leading not only to the transmission of mitogenic stimuli, but also to the activation of cellular pathways involved in neoplastic transformation as the RAS-MAPK or the PI3-kinase pathways (reviewed in Weintraub et al., 2013). Different studies regarding the role of EGFR in urinary bladder urothelial carcinomas have associated the overexpression of this growth factor receptor with poor prognosis, tumor stage and tumor progression (Chow et al.; Lipponen and Eskelinen, 1994; Mellon et al., 1995; Neal et al., 1990, 1985). In more recent studies the EGFR overexpression in urothelial carcinomas was not associated with mutations in the exons 18 to 21, which correspond to the tyrosine kinase domain (Chaux et al., 2012). These results were in accordance with a previous report, which stated that mutations within the tyrosine kinase domain were rare in bladder cancer (Blehm et al., 2006). Thus EGFR overexpression should have a different origin, such as gene amplification or upregulation of mRNA. A recent study has reported to have found *EGFR* gene amplification in 20% of the urothelial carcinomas (Millis et al., 2015). Previously an *EGFR* gene amplification rate of 4.6 % had been identified in the same type of tumors (Neal et al., 1990). Contrarily to what would be expectable, low mRNA levels of EGFR were associated with poor patient prognosis in a study which included non-invasive and muscle-invasive bladder tumors (Zaravinos et al., 2011). Nevertheless, the efficacy of EGFR inhibitors in urothelial carcinoma cell lines has been shown (Dominguez-Escrig et al., 2004; Nutt et al., 2004). This approach could be useful in patients with EGFR overexpression, though the results from clinical trials did not show any added benefit of this approach over the standard chemotherapy (Mooso et al., 2015).

Chapter II – Objectives

The main objective of this thesis was to contribute towards the progress of the knowledge concerning the role of BPV2 in the genesis of UBT in BEH-affected cattle, specifically using samples from bovine reared on the Azores Archipelago, the region of Portugal more severely affected. In order to do so, the experimental work was divided according to five specific objectives:

1 – To characterize the urinary bladder lesions used in the study.

2 – To determine the BPV2 prevalence in UBT.

3 – To assess BPV2's transcriptional activity in UBT through the detection of viral mRNA.

4 – To evaluate the cellular gene expression changes seen in UBT of BEH-affected cattle, and their possible association with BPV2 infection.

5 – To assess the effects of BPV2 oncoproteins, both in cell morphology and in gene expression patterns, using bovine kidney epithelial cells.

The following chapters are the outcomes of the studies conducted in order to achieve the above mentioned objectives.

Chapter III – Histopathological characterization of the bovine urinary bladder lesions, and the expression of cytokeratins in urothelial bladder tumors.

The contents of this chapter were partially presented as an oral communication in the 1st National Symposium Biomarkers in Animal Science and Veterinary Sciences - An Interdisciplinary Approach

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3.1 Abstract

Bovine Enzootic Hematuria is a chronic disease characterized by the intermittent presence of blood in urine and by the development of neoplasms in the urinary bladders of cattle, due to prolonged consumption of bracken. These neoplasms that arise in the urinary bladder of BEH-affected cattle can assume variable macroscopic appearances, and can have different histological origins. Inflammatory lesions are commonly seen in BEH cases, although neoplasms predominate in the cows that are slaughtered in abattoirs. The objective of this study was to characterize the urinary bladder lesions found in cattle reared in the Azores Archipelago, a BEH endemic area. During the year of 2012, a total of 69 macroscopic urinary bladder lesions were collected in the two major slaughterhouses of the Azores Archipelago. The histological analysis revealed that the majority of lesions (64/69) were neoplastic, and the ratio between epithelial and mesenchymal tumors was 43 to 21 respectively. The most commonly seen histopathological type of tumor was the urothelial carcinoma (26), followed by the urothelial papilloma (8). In order to better characterize these urothelial tumors, the expression of CK7 and CK20 was assessed by immunohistochemistry. A CK7 positive and CK20 positive immunophenotype was found in 24 of the 27 urothelial neoplasms studied. The amount of cells expressing CK7 decreased with increasing pathological grade and stage of the urothelial tumors ($p<0.001$), whereas the decrease in the number of neoplastic cells expressing CK20 was associated only with increasing pathological grade ($p<0.001$) with no variation regarding stage. These results point out the variability in lesions that can be found in BEH, and support the importance of this disease in the comparative pathology framework.

3.2 Introduction

Urinary bladder tumors (UBT) are considered to be uncommon in most domestic animals, and there are few reports concerning tumors of the lower urinary tract in species other than dogs, cats and cows (Maxi and Newman, 2007). In dogs urinary bladder neoplasms account for 0.5-1% of all diagnosed tumors, and the incidence is even lower in cats (Maxi and Newman, 2007; Meuten, 2002; Meuten et al., 2004). Contrarily to what is seen in other species, the incidence of UBT in cattle can reach up to 25% of all animals in certain regions of the globe (Meuten et al., 2004). The high incidence of UBT on those specific regions is associated with the endemic growth of bracken on the farmlands, and therefore with a high incidence of BEH. Outside these endemic regions the prevalence of UBT in cattle detected in slaughterhouses ranges 0.01-0.1% (Meuten et al., 2004).

The development of hematuria and urinary bladder neoplasms in cattle are the consequence, at least partially, of long term consumption of bracken (Pamukcu et al., 1976). The carcinogenic activity of bracken is associated with ptaquiloside, bracken's major toxic compound with proven clastogenic, mutagenic and carcinogenic effects (Alonso-Amelot and Avendaño, 2002; Smith, 1997). The participation of BPV2 in the carcinogenesis process was proposed in 1992, as this agent was isolated from naturally occurring and experimentally induced UBT in cattle (Campo et al., 1992). The synergistic action of environmental toxins and a papillomavirus in the genesis of neoplastic lesions has drawn the attention of many researchers, and in the last few years the role of BPV2 and other BPVs has been extensively studied.

Since the late 1950's the histology of the lesions present in the urinary bladders of BEH-affected animals has been the objective of some studies. The variability, not only in size, number, but also in the structure of the urinary bladder lesions in BEH cases was demonstrated in 1976 in the first large scale study where several naturally occurring and experimentally induced BEH cases were studied (Pamukcu et al., 1976). These findings were later supported by other studies from different countries where BEH is a serious animal health problem, such as Australia, Brazil, China, Portugal and Turkey (Carvalho et al., 2006; McKenzie, 1978; Özkul and Aydin, 1996; Peixoto et al., 2003; Xu, 1992). Both epithelial and mesenchymal tumors, of different histopathological types, can be present in the same urinary bladder. Some researchers have reported a higher incidence of mesenchymal neoplasms in BEH-affected cattle (Özkul and Aydin, 1996; Xu, 1992),

while others reported that epithelial neoplasms were more frequently diagnosed (Carvalho et al., 2006; Pamukcu et al., 1976).

Despite the diverse results, the most commonly diagnosed epithelial-derived neoplasms are urothelial carcinomas, adenocarcinomas and squamous cell carcinomas, whereas the most common endothelial-derived tumors are hemangiomas and hemangiosarcomas (Carvalho et al., 2006; Peixoto et al., 2003). In the past decade a large scale histological study on BEH-associated urinary bladder lesions was conducted in São Miguel Island, on the Azores Archipelago, the Portuguese BEH endemic area (Carvalho et al., 2006). As a result of that study, a new UBT histopathological category was introduced: the papillary neoplasm of apparently low malignant potential (PNALMP).

The presence of cytokeratins is used to characterize epithelial cells, as they are expressed both in normal and neoplastic cells (Moll, 1998). Regarding epithelial-derived UBT, the expression profile of cytokeratins is often used to discriminate urothelial carcinomas from other epithelial-derived neoplasms. In human pathology the combined expression of CK7 and CK20 is frequently assessed to confirm the urothelial origin of carcinomas (Bassily et al., 2000). The expression of CK7 and CK20 has also been studied in feline and canine urothelial tumors in an attempt of immunophenotyping these neoplasms (Espinosa de los Monteros et al., 1999; Ramos-Vara et al., 2003). The combined expression of both these cytokeratins in bovine urinary bladder urothelial neoplasms is still fairly unknown.

The goal of the current study was to characterize the urinary bladder lesions from BEH-affected cattle reared in the Azores Archipelago, both histologically and phenotypically for CK7 and CK20, as they were going to be the basis of the subsequent studies to be undertaken in the context of the present thesis.

3.3 Materials and methods

3.3.1 Sample collection

The urinary bladder lesion sampling process was performed in the two major Azorean slaughterhouses located in São Miguel and Terceira Islands, during the year of 2012. A total of 51 Friesian dairy cows slaughtered in the referred abattoirs were sampled. The age of the sampled animals ranged from 2 to 14 years. The urinary bladders of 38 carcasses, rejected due to the presence of bladder lesions, were examined in order to determine the number, and the size of the lesions and to collect samples. During the same period 13 urinary bladders without lesions were also sampled for control purposes. As a result of the existence of multiple lesions in some of the bladders examined, a total of 69 isolated, non-confluent lesions were sampled. Additionally, samples of the iliac lymph nodes of 22 animals with macroscopic bladder lesions and of the 13 animals without bladders lesions were also collected.

Tissue fragments were fixed in 10% buffered formalin and processed for routine histopathology with embedding in paraffin. Three-micron sections were stained with hematoxylin and eosin.

3.3.2 Histological analysis

Histological analysis of the bladder lesions was performed according to the criteria established by the World Health Organization and based on publications of BEH-associated bladder lesions (Carvalho et al., 2006; Eble et al., 2004; Peixoto et al., 2003). The lesions were diagnosed by two different observers, and whenever the diagnosis differed sections were re-examined in order to achieve a consensus.

The urothelial neoplasms were classified according to the degree of tissue invasion as follows: Ta, non-invasive papillary tumor; T1, suburothelial tumor; T2, tumor invades muscle; T3, tumor invades the bladder serosa; and T4, tumor exceeds the bladder serosa. The urothelial tumors were also classified according to the degree of cell differentiation: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; and G4, undifferentiated. In order to allow comparisons, papillomas were classified as Ta, non-invasive, and as G0.

3.3.3 Immunohistochemistry, antisera validation and grading

The cytokeratin expression pattern of urothelial neoplasms was determined using different antibodies reacting with a single cytokeratin, either CK7 or CK20. The primary antibodies, antigen retrieval methods and detection system used are presented on Table 5. Normal urothelium served as internal control for all antibodies employed. The immunohistochemical reactions were given a score, ranging from 0 to 4 for each antibody used, according to the percentage of immunoreactive neoplastic cells as follows: 0, less than 5% of positive cells; 1, 5 to 25% of positive cells; 2, 25 to 50% of positive cells; 3, 50 to 75% of positive cells, and 4, 75 to 100% of positive cells. Negative controls were processed similarly to the test slides, but the primary antibody was omitted and replaced by phosphate-buffered saline.

3.3.4 Statistical analysis

To test the possible statistical association between the immunoreactivity score with each antibody used and malignancy grade (G0-G4) and invasiveness stage (Ta-T4) a 2-sided Ficher's exact test was performed. Statistical analysis was carried out using R-Stat version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The results were considered statistically significant when *p*-values were less than 0.05.

Table 5 – Antibodies used and technical details.

Antigen	Clone (Source)	Retrieval method	Time/Dilution	Detection System (Source)
CK7	18-0234, clone OV-TL12/30 (Zymed Laboratories, San Francisco, CA)	Microwave, 600 Watts, 20 min. in EDTA pH 9	60 min/ 1:50	NovoLink Polymer Detection System (Leica Microsystems, Buffalo Grove, IL)
CK20	M7019, clone Ks20.8 (DakoCytomation, Carpinteria, CA)			

3.4 Results

3.4.1 Macroscopical evaluation

The urinary bladders of the BEH-affected cows exhibited a considerable variability of lesions present, both in number and in appearance (Figure 7). In 20 urinary bladders only one lesion was present, whereas multiple visible lesions were present in 18 urinary bladders. These lesions ranged from a few millimeters up to 10 centimeters, and occasionally were associated to visible signs of hemorrhage. There was no apparent specific location for the lesions, as these were randomly distributed throughout the organ. In a naked eye evaluation, no metastasis were present in the iliac lymph nodes collected from animals with urinary bladder tumors.

3.4.2 Microscopical evaluation

After histopathological diagnosis the 69 macroscopic isolated, non-confluent urinary bladder lesions, were divided into three major categories: inflammatory lesions, non-neoplastic epithelial abnormalities and neoplastic lesions (Figure 8). The histopathological diagnosis of all lesions collected are presented in Table 6. In the histological evaluation of the iliac lymph nodes sampled there were no signs of urinary bladder tumor metastisation.

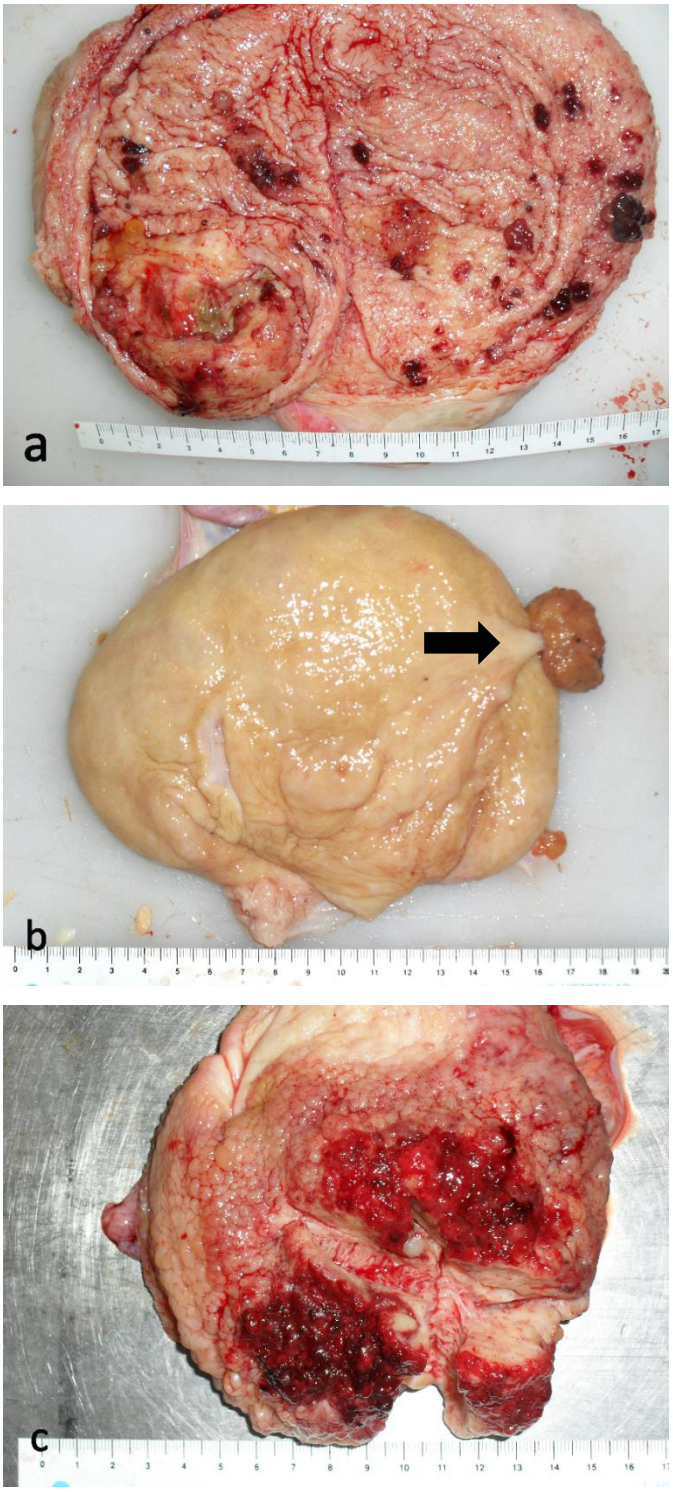

Figure 7 – Urinary bladder lesions. Bovine.

a – Multiple exophytic lesions disseminated throughout the urinary bladder, showing variable dimensions, some with associated hemorrhage.
b – Single exophytic papillary lesion with an evident stalk (arrow).
c – Extensive single infiltrating hemorrhagic lesion occupying most of the bladder luminal surface, thickening the urinary bladder wall.

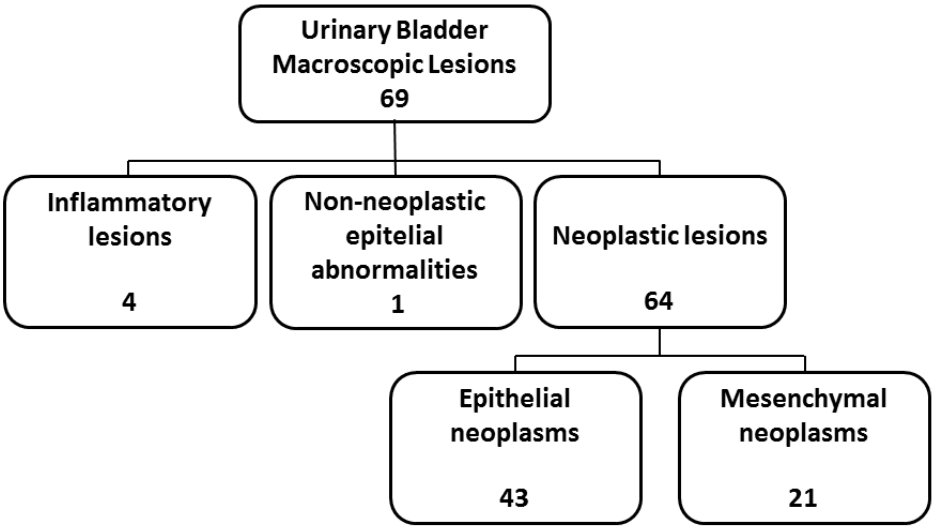

Figure 8 – Different types of histopathological lesions diagnosed in the urinary bladder samples.

3.4.2.1 Inflammatory lesions

Of the 69 macroscopic lesions collected four were inflammatory lesions which visibly distorted the bladder wall. Polypoid cystitis was observed in three of the cystitis cases and follicular cystitis was diagnosed in the remaining case. The cases of polypoid cystitis were characterized by finger-like protrusions of edematous areas of the lamina propria, often presenting congestion of the blood vessels, with areas of urothelial hyperplasia. Follicular cystitis was characterized by the presence of inflammatory cell aggregates (follicles), formed predominantly by lymphocytes and neutrophils, surrounding blood vessels in the lamina propria, with some degree of edema and congestion. In some areas the urothelium was invaded by these inflammatory cells, distorting the normal architecture of the epithelium.

3.4.2.2 Non neoplastic epithelial abnormalities

Glandular metaplasia resembling the intestinal mucosa, classified as intestinal metaplasia, was diagnosed in one of the urinary bladders collected. The urothelium formed multiple acini in the lamina propria which were lined by columnar epithelial cells and several goblet cells.

3.4.2.3 Neoplastic lesions

The vast majority of the lesions identified in the urinary bladders of cattle were neoplasms (64/69). From these 64 tumors, 43 were epithelial neoplasms and 21 were mesenchymal neoplasms.

3.4.2.3.1 Epithelial neoplasms

As mentioned above, the majority of the macroscopic lesion observed in the urinary bladders corresponded to epithelial neoplasms (43/69).

The most frequently diagnosed benign epithelial tumor (8/43) was the urothelial papilloma. These neoplasms were composed by variable numbers of occasionally ramified or exophytic fronds of fibrovascular stroma, arising from a delicate fibrous stalk, covered by epithelial cells identical to the normal urothelium (Figure 9 a). The number of cell layers, and the polarity of those cells was maintained when compared with the normal urothelium.

One adenoma was diagnosed (1/43), which was characterized by several exophytic projections, composed of multiple glandular structures covered by an epithelium similar to the urothelium. In some areas, these glandular structures were formed by a single layer of cuboid to squamous epithelial cells (Figure 9 b). Some of these structures were distended, forming cystic spaces, filled by variable amounts of eosinophilic amorphous material, sloughed epithelial cells and neutrophils. An inflammatory cell infiltrate, composed of lymphocytes, plasmocytes and neutrophils, was observed in the lamina propria underlying the neoplasm and surrounding some of the glandular structures.

Another type of exophytic neoplasm diagnosed was the papillary neoplasm of apparently low malignant potential (PNALMP) (2/43). These neoplasms showed some similarities with the urothelial papillomas, except for the number of cell layers present (Figure 9 c). Both of the PNALMPs diagnosed exceeded 5 cell layers of epithelial cells in thickness of the papillary fronds in some areas, indicating higher cellular proliferation when compared with urothelial papillomas. The epithelial cells maintained their polarity and few mitotic figures were observed. The thin fibrovascular stroma supporting the neoplastic epithelium was infiltrated by an inflammatory cell population mainly composed of lymphocytes, plasmocytes and neutrophils.

More than half of all epithelial tumors diagnosed were urothelial carcinomas (26/43). Some of these urothelial carcinomas showed a papillary exophytic growth pattern, with or without infiltration of the lamina propria (Figure 9 d and e), whereas others had an infiltrative growth pattern (Figure 9 f). Regarding the degree of invasion the urothelial carcinomas were classified according to the presence of neoplastic cells in the bladder layers: Ta (11/26), T1 (8/26), T2 (6/26), and T3 (1/26). The most common histological variants of urothelial carcinomas were the "nested" and the "microcystic".

Following the urothelial carcinoma, the adenocarcinoma was the second most frequent malignant epithelial neoplasm (4/32). These tumors often had both an exophytic and invasive growth pattern, infiltrating the lamina propria. Large cystic spaces could be occasionally observed (Figure 9 g). In some cases, within the same tumor the neoplastic glandular structures were covered either by columnar, cuboidal, transitional or goblet cells.

Finally, the squamous cell carcinoma was the less frequent malignant epithelial tumor diagnosed (2/32). These neoplasms were characterized by large groups of infiltrative neoplastic cells, forming nests or strands, where squamous differentiation of the epithelial cells resulted in the formation of keratin pearls, or keratin bundles (Figure 9 h). Glandular metaplasia was also seen in some areas surrounding the squamous cell carcinomas.

3.4.2.3.2 Mesenchymal neoplasms

The neoplasms of mesenchymal origin (21/64) diagnosed in the urinary bladders were all composed of neoplastic vascular tissue, presenting neoplastic blood vessels were localized in the lamina propria and presented either an endophytic or an exophytic growth pattern.

Hemangiomas (10/21) were characterized by the proliferation of clearly defined blood vessels filled with blood, covered by well differentiated endothelium (Figure 10 a). Two variants of hemangiomas, capillary (1/10) and cavernous (9/10), were observed.

Hemangiosarcomas (11/21), defined as malignant proliferations of endothelial cells, were formed by heterogeneous, irregular and anastomosing vascular spaces covered by atypical endothelial cells (Figure 10 b). Some areas of these tumors were solid, without visible vascular structures, and others were cystic. Both exophytic and endophytic growth patterns were observed. In the endophytic growing hemangiosarcomas, aggressive

invasive behavior was seen in some cases where the tumor (5/11) reached and invaded the muscle layers of the urinary bladder.

Table 6 – Histopathological types and numbers of urinary bladder tumors diagnosed.

Details of tumors	n	Percentage
Epithelial	43	67,2%
Benign		
Papilloma	8	12,5%
Adenoma	1	1,6%
PNALMP	2	3,1%
Malignant		
Urothelial carcinoma	26	40,6%
Adenocarcinoma	4	6,3%
Squamous cell carcinoma	2	3,1%
Mesenchymal	21	32,8%
Benign		
Hemangioma	10	15,6%
Malignant		
Hemangiosarcoma	11	17,2%
Total	64	100%

PNALMP – Papillary neoplasm of apparent low malignant potential.

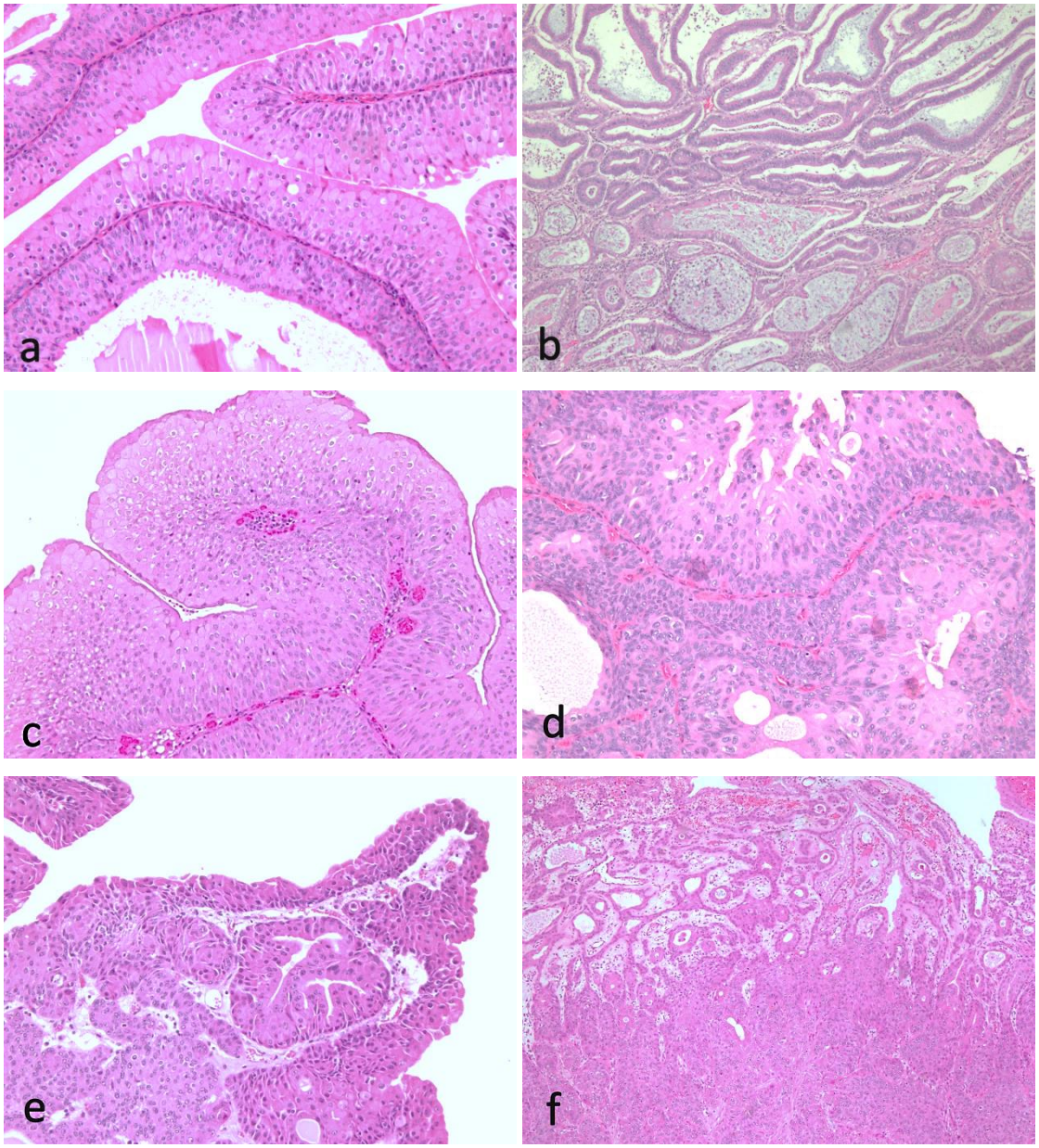

Figure 9 – Epithelial urinary bladder tumors. Bovine.
a – Urothelial papilloma. Finger-like projections are formed by a delicate fibrovascular stalks covered by normal-appearing urothelium. Hematoxylin and eosin, 100x.
b – Adenoma. The epithelial neoplastic cells form glandular structures in the lamina propria, covered by epithelial cells with glandular differentiation. Hematoxylin and eosin, 40x.
c – Papillary neoplasm of apparently low malignant potential. The finger-like projections are covered by an urothelium similar to the one found in urothelial papillomas, but with an increased number of cell layers. Hematoxylin and eosin, 100x.
d – Papillary and non-infiltrating urothelial carcinoma. The papillary fronds are formed by a thin fibrovascular stalk, covered by neoplastic urothelium with evident cellular atypia of the most superficial layer, without invasion of the supporting stroma. Hematoxylin and eosin, 100x.
e – Papillary and infiltrating urothelial carcinoma. A non-polarized urothelium covers the papillary frond, and the lamina propria is invaded by neoplastic urothelial cells. Hematoxylin and eosin, 100x.
f – Infiltrating urothelial carcinoma. The neoplastic urothelium deeply invades the lamina propria, forming several lobules of malignant cells closely packed together. Hematoxylin and eosin, 40x

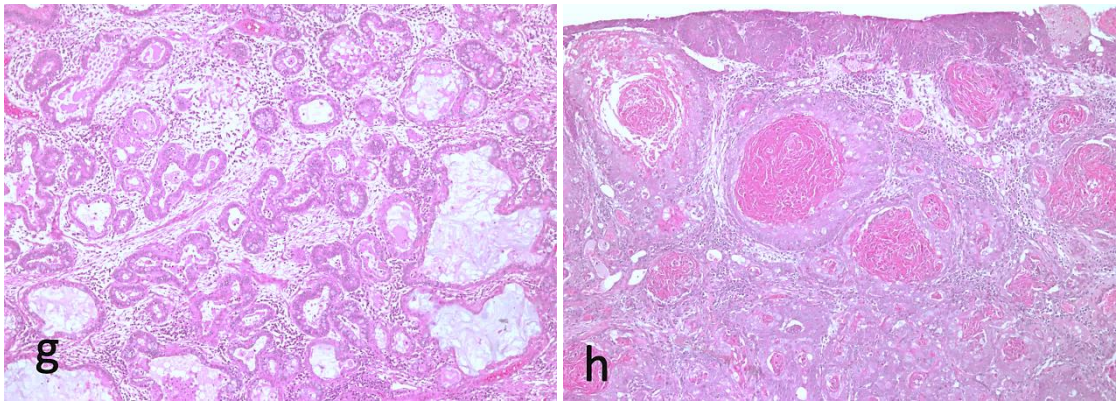

Figure 9 (continued).
g – Adenocarcinoma. Multiple irregular tubular structures, with variable dimensions, invading the lamina propria. Hematoxylin and eosin, 40x.
h – Squamous cell carcinoma. The neoplastic epithelium that invades the bladder stroma shows keratinization of the central areas of the lobules of neoplastic cells. Hematoxylin and eosin, 40x.

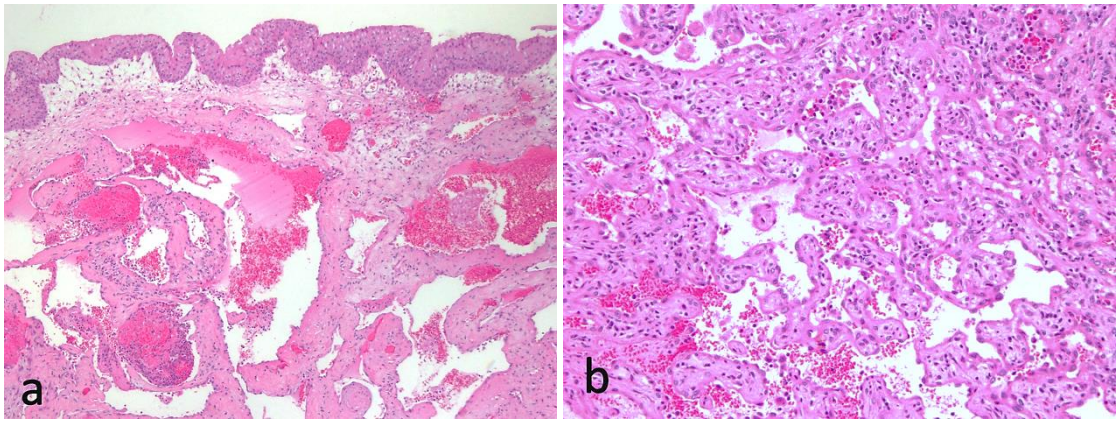

Figure 10 – Mesenchymal urinary bladder tumors. Bovine
a – Hemangioma. Several vascular structures, with well differentiated endothelium filled with variable amounts of blood cells occupy the lamina propria. Hematoxylin and eosin, 40x.
b – Hemangiosarcoma. Tumor mass is formed by irregular and, in some cases, anastomosing vascular spaces, covered by atypical endothelium. Hematoxylin and eosin, 100x.

3.4.4 Expression of cytokeratins in urothelial tumors

A total of 27 urothelial tumors, 6 urothelial papillomas and 21 urothelial carcinomas of the various grades and stages diagnosed from BEH-affected cattle, were selected (Table 7). This selection was carried out in order to achieve a representative sample of the urothelial tumors commonly seen in BEH cases. The urothelial tumors were divided in non-invasive (Ta) and invasive (T1-T3), and in low-grade (G1-G2) and high-grade (G3-G4) carcinomas.

Table 7 – Distribution of Grades and Stages of the urothelial tumors used for immunohistochemical studies.

Grade/Stage	G0	G1	G2	G3	G4	Total
Ta	6*	9	-	-	-	15
T1	-	-	5	1	-	6
T2	-	-	2	-	3	5
T3	-	-	-	1	-	1
Total	6	9	7	2	3	27

* Papillomas

3.4.4.1 CK7

In the urothelial papillomas studied, CK7 was found to be expressed in all the cell layers, with the exception of the most basal one, similarly to what was seen in normal urothelium (Figure 11 a). Generally, the staining was cytoplasmic and diffuse, with an increasing intensity observed from the lowest cell layers towards the most superficial ones. The weak staining of most of basal cells contrasted with the strong staining of most apical cells, which was stronger on the luminal surface of the cytoplasmic membrane of these cells. In other areas of the urothelial papillomas, a moderate to strong cytoplasmic staining of all the cell layers was observed, including the most basal ones, yet maintaining the intense staining pattern of the most superficial cells.

In low grade urothelial carcinomas the number of positive cells was reduced when compared with the normal urothelium, although maintaining, in most cases, the diffuse cytoplasmic staining, similar to the urothelial papillomas (Figure 11 c). In high-grade

urothelial carcinomas the expression of CK7 was lost in most of the neoplastic cells, with a heterogeneous distribution of positive staining within the tumor.

The expression of CK7 in non-invasive urothelial carcinomas was similar to the urothelial papillomas, regarding the staining pattern, but with fewer cells positively stained. In some papillary non-invasive neoplasms the number of cell layers stained was variable within the same tumor. In invasive carcinomas the number of positive cells, and the intensity of the staining was variable, as some groups of neoplastic cells maintained a strong and diffuse cytoplasmic staining, characteristic of the normal urothelium, surrounded by weakly stained or negative cells (Figure 11 e). The scores for CK7 and CK20 expression are displayed in Table 8. Overall, the number of CK7 positive cells gradually reduced with increasing grade ($p< 0.001$) (Table 9) and stage ($p< 0.001$) (Table 11).

3.4.4.2 CK20

The expression of CK20 in the urothelial papillomas was restricted to the cytoplasm of the most superficial cell layer of the urothelium in the majority of cases, similar to the expression pattern found in the normal urothelium (Figure 11 b). A diffuse cytoplasmic staining of all cell layers was seen in one urothelial papilloma, with stronger staining of the most superficial cells. Generally, the staining intensity observed was weaker to the one found for CK7 (Table 8).

In general, expression of CK20 in low-grade urothelial carcinomas was observed in less than 50% of the cells in all cases. The staining intensity was heterogeneous, varying from weak to strong, mostly confined to the most superficial cells, in the case of exophytic papillary tumors, or to the innermost cells of the lobules or groups of neoplastic cells in invasive tumors (Figure 11 d). In high-grade carcinomas the few CK20 positive cells present showed a staining pattern similar to the one described for the low-grade carcinomas.

The number of CK20 positive neoplastic cells in both invasive and non-invasive urothelial carcinomas was lower when compared with the urothelial papillomas. In some muscle invasive carcinomas the staining of the most superficial cells was maintained in the lobules of invasive neoplastic cells which formed small luminal cavities (Figure 11 f). Loss of CK20 expression was associated with increasing tumor grade ($p < 0.001$) but not with increasing tumor stage (Table 10 and 12).

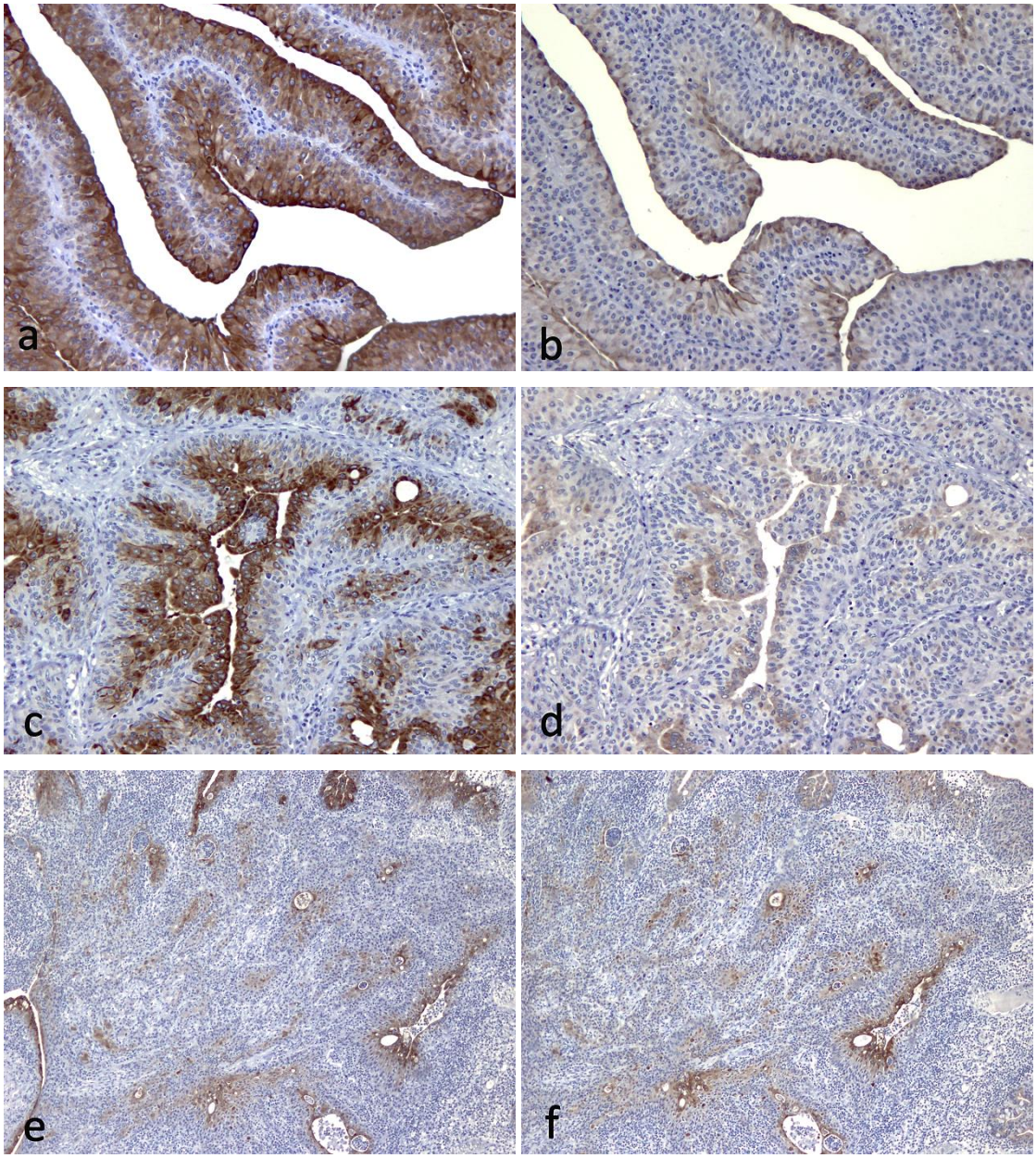

Figure 11 – Immunohistochemical detection of CK7 and CK20 in urothelial neoplasms of the urinary bladder. Bovine.

Urothelial papilloma. **a -** Intense and diffuse cytoplasmic CK7 staining of the majority of the cells layers. **b** – Expression of CK20 is restricted to the most superficial cells of the urothelium. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 100x.

TaG1 urothelial carcinoma. **c** - Although intense, the CK7 expression was detected in fewer neoplastic cells when compared with the urothelial papillomas. **d –** TaG1 urothelial carcinoma, urinary bladder, bovine. Irregular CK20 expression of the most superficial cells. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 100x.

T1G2 urothelial carcinoma. **e** - CK7 expression was mainly detected in the central areas of the lobules of infiltrative neoplastic cells. **f –** T1G2 urothelial carcinoma, urinary bladder, bovine. The CK20 immunostaining pattern was very similar to the one found with CK7 in this infiltrative carcinoma, although less intense in some areas. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 40x.

Table 8 – Immunohistochemical results obtained in the bovine urothelial neoplasms

Sample Number	Classification	CK7 Score	CK20 Score
1	Papilloma	4	4
2	Papilloma	4	3
3	Papilloma	4	3
4	Papilloma	4	2
5	Papilloma	4	3
6	Papilloma	4	3
7	UC TaG1	3	0
8	UC TaG1	3	2
9	UC TaG1	4	2
10	UC TaG1	2	2
11	UC TaG1	3	2
12	UC TaG1	3	2
13	UC TaG1	4	2
14	UC TaG1	3	2
15	UC TaG1	2	2
16	UC T1G2	2	2
17	UC T1G2	2	1
18	UC T1G2	0	0
19	UC T1G2	2	1
20	UC T1G2	2	2
21	UC T1G3	1	1
22	UC T2G2	2	2
23	UC T2G2	2	2
24	UC T2G4	1	1
25	UC T2G4	0	0
26	UC T2G4	1	1
27	UC T3G3	3	2

UC – urothelial carcinomas.

Table 9 – Cytokeratin 7 immunoreactivity scores in relation to pathological tumor grade.

Grade/ CK7 Score	0	1	2	3	4	Total	*P*
G0	-	-	-	-	6	6	< 0.001
G1	-	-	2	5	2	9	
G2	1	-	6	-	-	7	
G3	-	1	-	1	-	2	
G4	1	2	-	-	-	3	
Total	2	3	8	6	8	27	

Table 10 – Cytokeratin 20 immunoreactivity scores in relation to pathological tumor grade.

Grade/ CK20 Score	0	1	2	3	4	Total	*P*
G0	-	-	1	4	1	6	< 0.001
G1	1	-	8	-	-	9	
G2	1	2	4	-	-	7	
G3	-	1	1	-	-	2	
G4	1	2	-	-	-	3	
Total	3	5	14	4	1	27	

Table 11 – Cytokeratin 7 immunoreactivity scores in relation to pathological tumor stage.

Stage/ CK7 Score	0	1	2	3	4	Total	*P*
Ta	-	-	2	5	8	15	< 0.001
T1	1	1	4	-	-	6	
T2	1	2	2	-	-	5	
T3	-	-	-	1	-	1	
Total	2	3	8	6	8	27	

Table 12 – Cytokeratin 20 immunoreactivity scores in relation to pathological tumor stage.

Stage/ CK20 Score	0	1	2	3	4	Total	*P*
Ta	1	-	9	4	1	15	N.S.
T1	1	3	1	-	1	6	
T2	1	2	2	-	-	5	
T3	-	-	1	-	-	1	
Total	3	5	13	4	2	27	

3.4.4.3 BPV2 infection and Cytokeratin expression

The presence of BPV2 DNA found in some of the urinary bladder epithelial tumors used in this study (presented in detail in the following chapter) did not seem to affect the pattern of expression of either CK7 or CK20.

3.5 Discussion

This work confirms the wide range of histologically different lesions that can be found in the urinary bladders of BEH-affected cattle in the Azores Archipelago. Although using a smaller sample size, compared with large scale studies performed previously, the spectrum of urinary bladder lesions was similar, to some extent, to the one reported by other researchers (Carvalho et al., 2006; Özkul and Aydin, 1996; Pamukcu et al., 1976; Peixoto et al., 2003; Xu, 1992). The purpose of this analysis was also to characterize clearly the sample used in the subsequent work.

Only a small portion of the urinary bladder macroscopic lesions corresponded to inflammatory or non-neoplastic abnormalities 7.2 % (5/69), and these lesions were present in urinary bladders without any detectable bladder tumors (three of a total of 38 urinary bladders with macroscopic lesions). In the extensive work of Carvalho *et al*. (2006), the amount of urinary bladders analyzed with non-neoplastic lesions, accounted for 12.7% (55/433).

Regarding the UBT diagnosed, the ratio between epithelial and mesenchymal tumors, and the percentages of each histopathological type were quite similar to the previously mentioned study performed using urinary bladders from São Miguel Island, Azores (Carvalho et al., 2006). In the present study, epithelial-derived tumors accounted for nearly 70% of all tumors diagnosed. The urothelial carcinoma was by far the most frequent tumor in the urinary bladder of BEH-affected (40.6%), supporting the results from previous reports (Carvalho et al., 2006; Pamukcu et al., 1976). These urothelial carcinomas presented different grades of cellular differentiation, ranging from well differentiated to anaplasic carcinomas, and variable degrees of local tissue invasion, from non-invasive to deeply invasive tumors, including one with clear infiltration of the bladder serosa. As expected, all of the non-invasive urothelial tumors were formed by

well differentiated neoplastic cells, whereas the invasive tumors were formed by poorly differentiated neoplastic cells, confirming that the invasive behavior seen in the bovine urothelial tumors is associated with poorly differentiated cells. The second most frequent epithelial neoplasm was the urothelial papilloma, preserving the normal urothelium architecture. The PNALMP histological type was also diagnosed among the urinary bladder lesions sampled. Whether if PNALMP is an intermediate stage of tumor progression between the urothelial papilloma and the non-invasive papillary carcinomas will remain as an ongoing question, since follow up studies are impossible to perform in BEH cases, as the vast majority of lesions is detected in the post-mortem examination in abattoirs.

In the present study, mesenchymal-derived tumors were limited to vascular tumors, hemangiomas and hemangiosarcomas. These vessel forming tumors were mainly exophytic, limited to the lamina propria, in the case of hemangiomas. On the contrary, in hemangiosarcomas they mostly assumed an infiltrative behavior, with muscle invasion in some cases. Other reported mesenchymal-derived tumors, such as fibromas, hemangioendotheliomas, fibrosarcomas or leiyomiosarcomas were not diagnosed in the urinary bladders sampled for the present study. This fact is certainly due to the smaller sample size used in this study, as the occurrence of the above mentioned tumors accounts for less than 2% of all the UBT diagnosed (Carvalho et al., 2006; Özkul and Aydin, 1996; Pamukcu et al., 1976). Still, the present results support the generally accepted ratio between epithelial and vascular neoplasms present in BEH cases, 60-70% and 30-40% respectively (Meuten et al., 2004). The exact mechanisms by which epithelial and vascular neoplasms initiate and progress are still unknwon, although the amount and duration of animal exposure to bracken's toxins can be major factors determining the frequency, nature and severity of the lesions found in BEH cases (Shahin et al., 1998b).

The combined expression of CK7 and CK20 was assessed in 27 urothelial neoplasms of the urinary bladder of BEH-affected cattle. In order to determine if pathological grade and/or stage influenced the expression of these cytokeratins, each urothelial neoplasm was scored according to the number of neoplastic cells expressing CK7 and CK20.

There was no difference between the expression of CK7 in urothelial papillomas and in the normal urothelium, however the staining pattern was progressively lost with increasing grade and stage, both in intensity and in the number of CK7 expressing cells. In some invasive carcinomas CK7 expression was completely absent. In these tumors

both grade and stage influenced the amount of cells expressing CK7 ($p<0.001$). These results confirm previous findings on the expression of structural proteins in urothelial neoplasms of the urinary bladder of cattle, where loss of CK7 expression was found to be associated with increasing pathological grade and stage (Cota et al., 2014). In humans a different expression pattern is commonly seen, since CK7 expression is maintained even in very infiltrative and undifferentiated urothelial carcinomas (Bassily et al., 2000; Chu et al., 2000) and, therefore, the expression of CK7 is helpful in determining the origin of such tumors.

In the present study, CK20 expression was restricted to the most superficial layer of the urothelium in urothelial papillomas, as is seen in the normal urothelium. Interestingly, this staining pattern was maintained even in the groups of neoplastic cells which limited overt luminal spaces in invasive tumors, in which the loss of CK20 expression in neoplastic cells was more evident than in CK7 in invasive tumors. This finding can be related with the ratio of normal urothelial cells which express CK7 or CK20, since most cell layers express CK7 and only the most superficial one expresses CK20 (Moll et al., 1988). The decrease in the number of CK20 expressing cells was associated with increasing grade ($p<0.001$) but not with tumor stage.

In a previous study, 75% of feline urothelial carcinomas had a CK7 and CK20 positive phenotype (Espinosa de los Monteros et al., 1999). In that same report, 50% of the canine urothelial carcinomas were CK7 and CK20 negative, suggesting that the loss of expression of those cytoskeletal proteins was a more frequent event in canine than in feline urothelial carcinomas (Espinosa de los Monteros et al., 1999). However, in a later study the results obtained were quite different, as CK7 was detected in almost every canine urothelial neoplasm, including papillomas and carcinomas, suggesting that, in dogs, CK7 expression is maintained despite the malignancy of the tumor (Ramos-Vara et al., 2003). Those authors also found that the loss of CK20 expression was more frequent in canine urothelial papillomas than in urothelial carcinomas, as only 14.3% of the urothelial papillomas expressed CK20, contrasting with 68.5% of CK20 positive urothelial carcinomas (Ramos-Vara et al., 2003). In the present report, all of the urothelial papillomas expressed both CK7 and CK20, and total loss of expression was also found in urothelial carcinomas. This study adds new information to our previous study on the expression of cytokeratins found in urothelial neoplasms of the urinary bladder of BEH-affected cattle (Cota et al., 2014).

In conclusion, these results demonstrate that BEH is a unique condition, where multiple neoplasms, with different characteristics, can arise within the same host. This variability is also patent in the preservation of cytoskeleton proteins, since different urothelial tumors with similar grade and stage presented diverse numbers of CK7 and CK20 expressing cells. Although the variations in the number of CK7 and CK20 positive cells were observed, simultaneous expression of CK7 and CK20 was found in 24 of 27 urothelial tumors studied. It seems that independently of the behavior that the urothelial neoplasms might assume these retain the expression of urothelial associated cytokeratins such as CK7 and CK20. These results suggest that these two cytoskeletal makers can be useful for immunophenotyping BEH neoplastic lesions, helping to understand the essential steps that occur in the oncological process found in BEH.

Chapter IV – Detection and quantification of bovine papillomavirus type 2 in urinary bladders and lymph nodes in cases of Bovine Enzootic Hematuria from the endemic region of Azores

The contents of this chapter were fully published in Veterinary Microbiology

4.1 Abstract

Bovine Enzootic Hematuria (BEH) is a disease with a severe impact on production indexes and characterized by the development of bovine urinary bladder tumors, particularly in the Azores archipelago. The purpose of this study was to investigate and quantify BPV2 tissue distribution in bovine urinary bladder tumors, normal bladders, and iliac lymph nodes of cattle from the Azores. A real-time PCR system targeting the L1 gene was developed and allowed for the specific detection of the virus. BPV2 DNA was detected in a large proportion of the samples tested, both from neoplastic and healthy tissues, indicating that this virus is very prevalent in the bovine population of the Azores. Moreover, all types of tissues tested were positive, confirming a wide viral distribution within the infected animal. Bovine cutaneous papillomas sampled from Portuguese mainland dairy cattle were used as controls. Viral load ranged between 2.2 $x10^4$ copies/cell in the skin papillomas, and 0.0002 copies/cell in the urinary bladders tumors from the Azores. This is the first report presenting quantitative data on BPV2 infection in bovine urinary bladder lesions from the Azores. This approach will provide a useful tool to evaluate the role of BPV2 not only in the pathogenesis BEH but also in cell transformation mechanisms.

4.2 Introduction

Papillomaviruses are small, non-enveloped, double-stranded DNA viruses that infect the epithelia of vertebrates. They cause the development of exophytic tumors, papillomas, in their natural hosts and in some cases in related species (de Villiers et al., 2004). Bovine papillomaviruses (BPV) are known to infect cutaneous and mucosal epithelia in cattle producing benign lesions. However, in the presence of environmental co-factors these lesions can progress to form malignant tumors in the urinary bladder and in the upper alimentary canal (Campo, 2006).

So far, thirteen BPV types have been described and their genomic sequences have been characterized. They belong to four genera, *Deltapapillomavirus* (BPV1, 2, and 13), *Epsilonpapillomavirus* (BPV5 and 8), *Xipapillomavirus* (BPV3, 4, 6, 10, 11 and 12) and *Dyoxipapillomavirus* (BPV7) (reviewed by Rector and Van Ranst, 2013).

Cattle grazing for long periods on bracken fern (*Pteridium aquilinum*)-infested pastures develop multiple tumors in the urinary bladder and hemorrhages of the mucosa, a syndrome called Bovine Enzootic Hematuria (BEH) (Carvalho et al., 2006; Meuten, 2004; Pamukcu et al., 1976). BPV2 infection and the ingestion of bracken fern have already been postulated as the conditions necessary for the development of these neoplastic lesions (Campo et al., 1992).

BPV1/2 DNA has been amplified from bovine urinary bladder samples collected in multiple BEH endemic regions of the globe, such as the Azores archipelago, Brazil, Italy, India and Romania (Balcos et al., 2008; Borzacchiello et al., 2003; Pathania et al., 2012; Resendes et al., 2011; Wosiacki et al., 2005). The BPV1/2 DNA detected in urinary bladder samples has also been identified in other tissues/body fluids such as the placenta, commercial frozen semen, milk, urine and blood (Lindsey CL, 2009; Pathania et al., 2012; Roperto et al., 2012; Roperto et al., 2008; Silva et al., 2011; Wosiacki et al., 2005).

The aim of this study was to detect and quantify virus distribution in tissues of cattle in the Azores archipelago, using a TaqMan™-based quantitative PCR (qPCR) system. Due to its sensitivity and reliability, this assay confirmed the prevalence of BPV2 among cattle in this geographic region and demonstrated a widespread tissue distribution within an individual host.

4.3 Materials and methods

4.3.1 Sample collection

During the year of 2012, the urinary bladders (UB) of 51 cows were sampled in two slaughterhouses of the Azores Archipelago. The age ranged from 2 to 14 years. Thirty-eight animals had macroscopic lesions in the UB and 13 showed no lesions. A total of 69 isolated, nonconfluent lesion samples (Table 13) were collected from the affected UBs plus 13 samples from normal UBs. In some of the UBs with visible lesions, multiple samples were also taken from areas with no gross changes. The iliac lymph nodes of 35 animals were sampled.

Nine cutaneous papillomas, ranging from 0.5 cm to 5 cm in diameter, were collected from cattle in the Portuguese mainland and used as positive controls of the natural BPV2 infection.

In all cases, sampling was performed using a set of disposable sterile scalpel blades and plastic tweezers, replaced for each sample. Fragments collected for molecular analysis were preserved in RNAlater® (Ambion) and maintained at 4ºC until DNA extraction was performed. For routine histopathology diagnosis, fragments were fixed in 10% buffered formaldehyde. Histological typing of the lesions was based on the World Health Organization established criteria and on publications of BEH-associated bladder lesions (Meuten, 2004; Peixoto et al., 2003). The classification of specific lesions such as PNALMP (papillary neoplasm of apparently low malignant potential) was based on the work from Carvalho et al., 2006.

4.3.2 DNA extraction

Total DNA extraction was carried out using the DNeasy® Blood and Tissue kit (Qiagen, Germany), according to the manufacturer's protocol. After determining the concentration and purity of the DNA using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA), the genomic DNA was stored at -80ºC.

4.3.3 Design of primers and probes

A conserved region within the BPV2 L1 gene was chosen as a suitable target for quantitative PCR (qPCR) detection of BPV2 DNA in bovine tissue samples. Conserved regions within L1 were identified by multiple alignments of the nucleotide sequences of different BPV2 isolates (available through GenBank) using the CLC Main WorkBench V6 application. A similar method was performed for BPV2 E5 gene. The primers and probe were designed using Primer3 software (Applied Biosystem) and purchased from Stab Vida Genomics Lab Portugal. Their sequences were: L1 forward primer, 5'-CTTATGCAGGGCTTAAGTTTTGGAGCATAG-3'; reverse primer, 5'-CAACAGCTCTCTTTCTCACAGTTGAACATC-3'; TaqMan™ probe, FAM 5'-CCCTTGGGAAGAAGATTCTTAGCTCAGCAAGGGGC-3'TAMRA; E5 forward primer, 5'- TCTATGGTTTCTATTGTTCTTGGGACTA - 3'; reverse primer , 5' – GAGCACTCAAAATGATCCCAGTATAC - 3'; TaqMan™ probe , JOE 5'-TGCTGCAATGCAACTGCTGCTGTTACT - 3' TAMRA.

4.3.4 Quantitative PCR

Screening and quantification of BPV2 was assessed by identification of the L1 gene by qPCR in a StepOnePlus^{TM} Real-time PCR system (Applied Biosystems, Foster City, USA), using the TaqMan^{TM} Real time PCR Master Mix (Applied Biosystems). All amplifications were performed in a 20 µl mixture volume per reaction, containing 0.9 pmol of forward primer, 0.9 pmol of reverse primer, 0.25 pmol of probe and 200 ng of sample DNA, the equivalent to 66000 bovine cells (Yuan et al., 2007). The cycling conditions included an initial denaturation step at 95ºC for 10 min followed by 50 cycles of denaturation at 90ºC for 15 sec, annealing at 56ºC for 20 sec and extension at 72ºC for 30 sec. The fluorescence detection was carried out in the extension step. All samples were performed in duplicate. Multiple negative controls lacking DNA template were used in each assay.

4.3.5 Plasmid construction

Using the above qPCR system, a 135-bp DNA fragment was amplified from multiple urinary bladder samples. One such amplicon was cloned into a pGEM®-T Easy Vector (Promega Corporation, Madison, USA), according to the manufacturer's instructions. After transforming *Escherichia coli* Dh5α competent cells with the recombinant plasmid, it was purified using the ZR Plasmid Miniprep™-Classic (Zymed Research, Irvine, USA). The concentration and purity of the plasmid DNA was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA), and the DNA was stored at -80 ºC until use. Sequencing of the plasmid revealed that the insert retained identity with the BPV2 L1 gene.

In order to estimate the copy number of the BPV2 genome in each sample, a standard curve was generated using ten-fold serial dilutions of purified recombinant L1 plasmid DNA and the StepOne™ software v2.3.

4.3.6 E5 and L1 expression analysis

To investigate the transcriptional activity of BPV2 in the positive urinary bladder samples, reverse transcription (RT)-PCR was performed. First, total RNA was extracted from BPV2-positive urinary bladder samples as well as 9 cutaneous papillomas, which served as positive controls, using RNeasy® Mini kit (Qiagen, Germany), according to the manufacturer's protocol. The RNA concentration and purity was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA) and was stored at -80 ºC until use. Approximately one microgram of RNA from each sample was treated with DNase I (Invitrogen, USA) to eliminate any viral DNA present and then subjected to reverse transcription using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland) with anchored Oligo (dT). Detection of BPV2's E5 and L1 was assessed by qPCR in a 7300 Real-time PCR system (Applied Biosystems, Foster City, USA), using the TaqMan™ Real time PCR Master Mix (Applied Biosystems, Foster City, USA). All amplifications were performed in a 20 µl mixture volume per reaction, containing 0.9 pmol of forward primers, 0.9 pmol of reverse primers, 0.25 pmol of the probes and 100 ng of cDNA as template using the cycling conditions were mentioned above. The fluorescence detection was carried out in the extension step. All samples were performed

in duplicate. Multiple negative controls lacking cDNA template were included in each assay.

4.3.7 Statistical analysis

Association between the presence/absence of BPV2 DNA and the type of urinary bladder lesion diagnosed (epithelial tumors, mesenchymal tumors, inflammatory and non-neoplastic lesions) was evaluated using a 2-sided Fisher's exact test. Variance in viral loads detected in epithelial tumors, mesenchymal tumors, inflammatory and non-neoplastic lesions, and in normal UBs were analyzed using one-way ANOVA. Statistical analysis was performed using R-Stat version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria). The results were considered statistically significant when *p*-values were less than 0.05.

4.4 Results

4.4.1 Specificity and sensitivity of the qPCR system

The qPCR system amplified a 135 bp fragment in multiple urinary bladder samples. Direct sequencing of the135bp-amplicons revealed that all shared 98-100% sequence identity with BPV2's L1 gene corresponding to nucleotides 6910-7044 within the BPV2 genome (GenBank accession number M20219-1). This confirmed the specificity of the qPCR assay and its suitability for detecting BPV2 DNA in tissue samples.

One of the L1 amplicons was cloned into the pGEM vector, and the resulting recombinant L1 plasmid DNA was used to determine the sensitivity and efficiency of the qPCR system. Performing the qPCR assay on serial dilutions of the plasmid DNA revealed that the detection limit of the qPCR system was only 10 target copies. This also established a standard curve for determining DNA copy number in bovine tissue samples. To assess the variation of the Cycle Threshold (Ct) values and reaction efficiencies between assays, tenfold serial dilutions of the recombinant L1 plasmid DNA were tested repeatedly. The correlation efficiency (R-squared) value was 0.996 ± 0.002, and the average slope value was – 3.504 ± 0.169, corresponding to an estimated assay efficiency of 92.9%. Thus, the qPCR assay developed here displayed high sensitivity and efficiency when tested against a recombinant DNA target.

4.4.2 BPV2 in the animals with UB lesions

Of the 38 BEH affected animals, 27 (71%) were positive for BPV2 DNA in at least one of the types of tissue samples tested (Figure 12). Nineteen of these animals had one or more UB lesions positive for BPV2 DNA. In five of the animals, viral DNA was found only in the normal areas of the UB surrounding the lesions. Three of the 38 animals were positive for BPV2 DNA only in the iliac lymph nodes.

From a total of 69 UB lesions, BPV2 DNA was detected in 28 of them (41%). Viral loads in these lesions varied from 0.00017 to 0.072 viral DNA copies/cell (Table 14). The histological type of the BPV2-positive lesions included 7 urothelial carcinomas, 7 hemangiosarcomas, 5 hemangiomas, 4 urothelial papillomas, 1 papillary neoplasm of apparently low malignant potential (PNALMP), 1 squamous cell carcinoma, 1 adenocarcinoma, 1 intestinal metaplasia and 1 cystitis (Table 13). BPV2 DNA was present in more of the mesenchymal tumors (57%) than in epithelial ones (33%), although there was no statistically significant association between the presence of BPV2 DNA and a particular histological type or the viral load.

Table 13 – Histopathological types of the lesions sampled in the slaughterhouses of the Azores Archipelago

Details of lesion samples	Number of samples	Number of BPV2 positive samples
Total epithelial tumors	43	14
Urothelial carcinoma	26	7
Squamous cell carcinoma	2	1
Adenocarcinoma	4	1
Urothelial papilloma	8	4
PNALMP	2	1
Adenoma	1	0
Total mesenchymal tumors	21	12
Hemangiosarcoma	11	7
Hemangioma	10	5
Total inflammatory and non-neoplastic lesions	5	2
Cystitis	4	1
Intestinal metaplasia	1	1

Abbreviation: PNALMP, papillary neoplasm of apparently low malignant potential

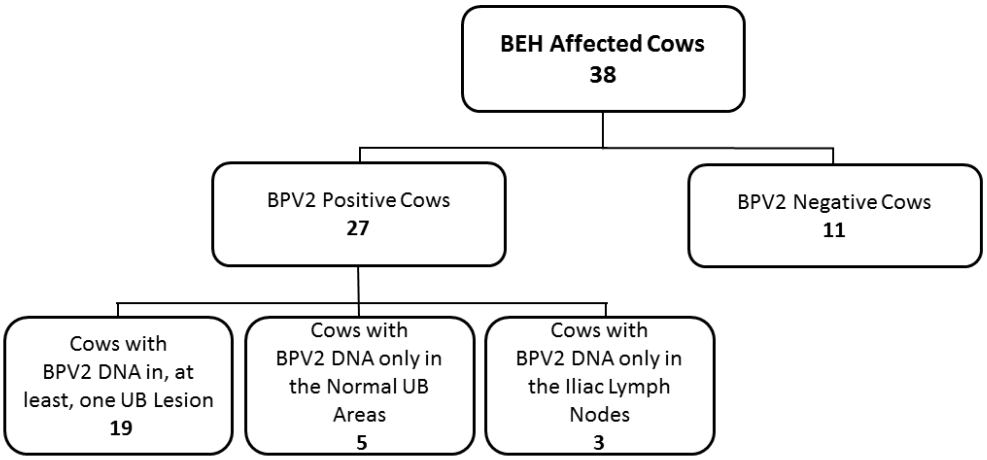

Figure 12 – Detection of BPV2 DNA in BEH affected cows.

4.4.3 BPV2 in animals without UB lesions

The UBs from 6 out of 13 (46%) control animals were positive for BPV2 DNA, with viral loads ranging from 0.00087 viral DNA copies/cell to 4.2 viral DNA copies/cell (Table 14). One of the control animals was only positive in the iliac lymph node. No microscopic alterations were observed in the UB samples of any of the control animals.

4.4.4 BPV2 in animals with cutaneous papillomas

All of the 9 cutaneous papillomas (100%) were positive for BPV2 DNA, with viral loads ranging from one copy of BPV2 DNA per cell to a maximum of 2.2 $x10^4$ copies/cell ($\acute{x}$=10^3 copies/cell) (Table 14). Histologically these lesions were diagnosed as fibropapillomas, with a significant mesenchymal component, rich in fibroblasts, underlying the epithelial component of the tumor.

Table 14– Results of BPV2 quantification by qPCR in different bovine tissue samples

Type of sample	Number of samples	Number of BPV2 positive samples (%)	Lowest copy/cell number	Highest copy/cell number	Mean copy/cell number
Urinary bladder Lesions	**69**	**28 (41%)**	**0.00017**	**0.072**	**0.0072**
Normal urinary bladders	**13**	**6 (46%)**	**0.00087**	**4.20**	**0.70**
Iliac lymph nodes	**35**	**9 (26%)**	**0.00039**	**0.011**	**0.0038**
Cutaneous Papillomas	**9**	**9 (100%)**	**1.1**	**21760**	**4440**

4.4.5 E5 and L1 gene expression in urinary bladder samples

No amplification of E5 or L1 gene transcripts from cDNA templates was detected in any of BPV2 positive urinary bladder samples (neoplastic, inflammatory and normal samples). Both E5 and L1 mRNAs were detected in all of the 9 cutaneous papillomas.

4.5 Discussion

BPV-associated lesions still remain an important cause of economic losses both in cattle and in equids. In cattle, such losses are connected with the various forms of BPV-induced lesions, including skin, teats and penis papillomas, gastrointestinal and UB neoplasms, with severe economic impact due to direct production losses related to carcass rejections and government compensations.

In the present study, the development of a specific and sensitive Taqman™ based qPCR system allowed the detection and quantification of BPV2 in different tissue types of healthy and BEH-affected cattle in the Azores, permitting an insight into the viral distribution within the host.

Various groups have identified BPV2 DNA in urinary bladder lesions of cattle using non-quantitative conventional PCR systems (Balcos et al., 2008; Borzacchiello et al., 2007; Resendes et al., 2011; Roperto et al., 2005; Wosiacki et al., 2005; Wosiacki et al., 2006). Reports on viral loads present in the UB lesions were limited to two. First, Yuan et al, 2007, described the use of an E5-specific TaqMan™ system for detection and quantification of BPV1 and BPV2 in both bovine urinary cancers and equine sarcoids. But, de Villiers et al., 2004, showed that the L1 ORF and not E5 is the preferred target sequence for identification of PV type. Second, Pathania et al, 2012, reported the development of a SyberGreen (SYB) qPCR assay based on the L1 gene for BPV2 detection and viral load determination in UB lesions. However, the use of a SYB qPCR relies on the analysis of amplicon melting curves, and nucleotide diversity may induce changes in the amplicon melting temperature. In our study, the use of a TaqMan™-based system targeting BPV2's L1 gene was therefore paramount for the specificity of the assay when using bovine tissue samples, and, due to TaqMan™ chemistry, ambiguities of the results were reduced. The high specificity and sensitivity of the assay was well demonstrated since all positive amplicons displayed 98-100% identity with BPV2 L1 gene and as few as 10 molecules of BPV2 could be detected. This feature is essential for

the detection of natural UB infection in cattle, since we found that the average viral loads in the UB samples tested were below 1 copy/cell, similar to other published results (Pathania et al., 2012; Yuan et al., 2007).

The variation observed in the viral loads of epithelial and mesenchymal tumors, non-neoplastic lesions and control UBs tested were not statistically different. These results are in accordance with the suggestion that the infection of UB epithelial and mesenchymal cells by BPV2, is an abortive one, with no production of viral particles (Campo et al., 1992; Yuan et al., 2007). The thousands of viral genome copies/cell detected in the cutaneous papillomas contrasts with what was found in UB tissues, suggesting that the urothelium lacks the conditions present in the skin that are required for a productive BPV infection. Consistent with this notion, we were unable to detect BPV2 E5 or L1 gene transcripts in the samples of urinary bladders (with or without lesions) using a highly sensitive qRT-PCR system. The lack of E5 expression even in UB tumors could be explained by an abortive infection in which E5 is expressed only early during infection and plays a role in initiation but not maintenance of neoplastic transformation. Others have reported detection of E5 and L1 proteins by immunohistochemistry and western blot not only in naturally occurring bladder tumors of cattle and water buffalos (Roperto et al., 2013), but also in the placenta of cows with urinary bladder tumors (Roperto et al., 2012) and in peripheral blood mononuclear cells (Roperto et al., 2011). The disparity between these previous reports and our results might be related to differences in the assays used (protein versus mRNA detection), methods of sample collection and/or variation in host animals from different geographic regions. Further studies are required to better understand the capability of BPV2 to replicate in the urothelium. It is important to note that the presence of BPV2 in the iliac lymph nodes is evidence of BPV2's ability to infect non-epithelial cells. This is consistent with a report suggesting that systemic dissemination of virus through the blood stream might allow it to reach several organs including the urinary bladder (Roperto et al., 2011).

Importantly, we show here that a substantial number (41%) of UB lesions tested positive for BPV2 DNA. Several additional UB lesions displayed BPV2 DNA in the surrounding tissue, which may indicate a transient presence of BPV2 in the lesion. We also found that a greater percentage of mesenchymal UB lesions harbored BPV2 DNA compared with epithelial ones. However, no statistically significant association between a specific lesion type and BPV2 DNA presence was observed, consistent with previously reported findings (Balcos et al., 2008; Borzacchiello et al., 2007).

A study carried out in Brazil found a significant association between BPV2 diagnosis and urinary bladder macroscopic lesions (Wosiacki et al., 2006). According to this report, only 10% of the control animals with no UB lesions harbored BPV2 DNA. In contrast, we detected BPV2 DNA in 46% of the control animals as well as in normal areas surrounding the UB lesions. This apparent discrepancy could be explained by factors that favor transmission of BPV2 in animals living in the Azores, such as their geographical isolation on islands and/or their genetic background. Nonetheless, we still demonstrated a higher incidence BPV2 infection in BEH affected animals, 71% compared with 46% in the control animals. Furthermore, although the control animals displayed no signs of neoplasia, those that harbor BPV2 may be predisposed to developing UB tumors in the future. Taken together, our results suggest that the widespread presence of BPV2 in cattle in the Azores may play a necessary but insufficient role in UB neoplasia.

In summary, this is the first report on the quantification of BPV2 DNA in UBs of cattle from the Azores archipelago, a BEH endemic region. The overall number of BPV2 positive animals indicates that this agent is widespread in the bovine population of the archipelago, making it a good *in vivo* model to better understand the PV mechanisms of cell transformation. BPV2 DNA was found present in UB tumors as well as control animals without UB lesions, suggesting that BPV2 is not sufficient for neoplastic transformation. Importantly, the high sensitivity, specificity and efficiency of the developed qPCR system allowed the detection of BPV2 DNA in more than one tissue type, revealing it to be a useful tool in the study of BPV infection.

Chapter V – Gene expression of cell cycle regulators and epithelial growth factor receptor in BPV2 positive urinary bladder tumors of Bovine Enzootic Hematuria-affected cattle

The contents of this chapter were partially presented as poster communication in the HPV 2015, 30th International Papillomavirus Conference & Clinical and Health Workshops

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5.1 Abstract

Bovine Papillomavirus type 2 (BPV2) has been associated with a naturally occurring neoplastic syndrome affecting cattle that graze in bracken fern infested pastures named Bovine Enzootic Hematuria (BEH). Frequently multiple tumors of different histological origins develop in the urinary bladder of these animals. The aim of this study was to assess the expression of cell cycle controlling genes *TP53*, *MDM2* and *CCND1*, and of the *EGFR* gene in BPV2 positive urinary bladder epithelial and endothelial tumors. The effects of BPV2 in commonly altered pathways in neoplastic lesions was evaluated by comparison of the expression profile of these genes with the one identified in BPV2 negative urinary bladder tumors. TP53, MDM2 and CCND1 were found to be overexpressed in most of the tumors studied, but no significant differences were seen between BPV2 positive and negative lesions. EGFR gene expression levels were significantly lower in BPV2 positive tumors when compared with BPV2 negative ones. This work provides an insight into the molecular events that take place in naturally occurring urinary bladder tumors in BEH, adding further knowledge on the BPV2 participation in this neoplastic syndrome.

5.2 Introduction

Urinary bladder tumors are rare in cattle, except in certain regions of the globe where bracken fern (*Pteridium aquilinum*) is common in the pastures where cattle graze (Meuten, 2002). The chronic ingestion of bracken fern by the animals leads to the development of BEH, a neoplastic syndrome which main feature is the development of single or multiple tumors in the urinary bladders of the affected animals (Campo et al., 1992; Carvalho et al., 2006; Meuten et al., 2004; Pamukcu et al., 1976). These tumors can have an epithelial or endothelial origin, the urothelial carcinoma being the most common neoplasm found in BEH cases (Carvalho et al., 2006; Peixoto et al., 2003).

The carcinogenic effect of bracken is mainly related with the presence of ptaquiloside (Pta), an unstable glycoside with potent carcinogenicity (Hirono et al., 1984b). Pta is an alkylating agent capable of activating proto-oncogenes as H-ras (Prakash et al., 1996) and breaking DNA strands after forming DNA adducts (Ojika et al., 1989).

The role of the BPV in the development of bovine urinary bladder tumors was first suggested in the 1950's (Olson et al., 1959), but it was much later that the synergistic association between the bracken's carcinogens and this infectious agent was experimentally reproduced (Campo et al. 1992). Since then various groups have reported the presence of BPV1/2 DNA in urinary bladder lesions of cattle from BEH endemic regions as the Azores, Brazil, India, Italy and Romania (Balcos et al., 2008; Borzacchiello, 2003; Cota et al., 2015; Pathania et al., 2012; Resendes et al., 2011; Wosiacki et al., 2005). Recently BPV13, the novel Deltapapillomavirus (Lunardi et al., 2013a), was identified in urothelial bladder tumors of cattle grazing in bracken fern infested pastures of southern Italy (Roperto et al., 2015).

Changes at the molecular level of the cell cycle controlling genes are central steps in the carcinogenic process of the urinary bladder (Yurakh et al., 2006). Deletions or mutations of the *TP53* tumor suppressor gene are found in 70% of cases of muscle invasive bladder cancer (MIBC) in man (Goebell and Knowles, 2010). Such gene mutations, which are mainly point-mutations, give rise to functionally inactive p53 proteins that accumulate in the nucleus of tumor cells, due to their increased stability when compared with the wild-type p53 proteins (Vousden and Lu, 2002). Abnormal p53 expression has been reported both in epithelial and vascular tumors of the urinary bladders of BEH-affected cattle suggesting alterations in the *TP53* gene (Carvalho et al., 2009; Cota et al., 2014). The p53

function can also be suppressed by cellular proteins such as murine double minus-2 protein (MDM2). MDM2 negatively regulates p53 by binding to it and leading to proteasomal degradation (Zhao et al., 2014). *MDM2* gene amplifications have been describe to occur in urothelial carcinomas (Goebell and Knowles, 2010; Ross et al., 2014), but there have not been any studies on the expression of this gene in bovine urinary bladder tumors. *CCND1* gene encodes the Cyclin D1 protein, a nuclear cell-cycle regulating protein required for the G1/S phase transition (Baldin et al., 1993). *CCND1* gene amplifications are found in 10-20% of all cases of muscle invasive bladder cancer (Goebell and Knowles, 2010). The Cyclin D1 cell cycle regulatory pathway was reported to be altered in BEH associated urinary bladder tumors (Carvalho et al., 2009; Cota et al., 2014). The epidermal growth factor receptor (EGFR) belongs to the ErbB family of tyrosine kinases and its abnormal expression has been reported in carcinomas arising from several organs including the urinary bladder (Yarden and Sliwkowski 2001). Furthermore EGFR is not only expressed in epithelial cells as it was found to be expressed in endothelial tumor cells (Amin et al., 2006).

The goal of the present study was to investigate the expression of cell cycle regulators and of *EGFR* by quantitative gene expression methods in a series of BPV2 positive and negative epithelial and endothelial tumors of the urinary bladder of BEH-affected cattle. By comparing the groups of BPV2 positive/negative tumors we sought to assess if the presence or absence of BPV2 affects the pattern of expression of the genes studied.

5.3 Materials and methods

5.3.1 Tissue samples and histological typing

All bladder samples were collected in the two major slaughterhouses of the Azores Archipelago during the year of 2012 within the scope of a previous work regarding BPV2 detection in urinary bladders of BEH-affected cattle (Cota et al., 2015). A total of 51 cows were sampled. The ages ranged from 2 to 14 years. Briefly, part of the tissue samples was collected for molecular analysis, preserved in RNAlater® (Ambion®, Life Technologies) and maintained at 4ºC until nucleic acid extraction was performed. The remaining portion of each tissue sample was fixed in 10% buffered formaldehyde for routine histopathology diagnosis and immunohistochemistry.

After paraffin embedding, three-micron thick sections of each tissue sample were stained with hematoxylin and eosin. Histological typing of the samples was based on the WHO established criteria and on publications of BEH-associated bladder lesions (Meuten, 2004; Peixoto et al., 2003; Carvalho et al., 2006).

Upon histological diagnosis and BPV2 DNA detection using Taqman™-based quantitative PCR system described in (Cota et al., 2015) tumors of epithelial and endothelial origin were selected, resulting in a total of 38 samples (Table 15). Five BPV2 negative normal urinary bladder samples were used as controls. The samples were grouped by histopathological type and by BPV2 status.

Table 15 – Number of samples per histological type and BPV2 status

Diagnosis	BPV2 positive	BPV2 negative	Total
Urothelial papilloma	4	3	7
Urothelial carcinoma	7	7	14
Hemangioma	5	4	9
Hemangiosarcoma	5	3	8

5.3.2 Design of Primers

Primers for *TP53*, *MDM2*, *CCND1* and *EGFR* gene expression analysis were designed using Primer Express software (Applied Biosystems, USA) and purchased from Stab Vida, Portugal. The primers were designed in the exon-exon junction areas of the genes and their sequences were submitted to the Blast software, available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov), in order to evaluate the sequence specificity. The sequences of the primers used are disclosed in Table 16.

5.3.3 RNA extraction, reverse transcription and quantitative gene expression analysis (qPCR)

Total RNA was extracted from the urinary bladder samples RNeasy® Mini kit (Qiagen, Germany), according to the manufacturer's protocol. The RNA concentration and purity was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA)

and was stored at -80 ºC until use. Approximately one microgram of RNA from each sample was subjected to reverse transcription using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland). Amplification of the mRNA was performed using StepOnePlusTM Real-time PCR system (Applied Biosystems, USA) and the Luminaris Color HiGreen High ROX qPCR Master Mix (Life Technologies, USA). All amplifications were performed in a 12.5 µl mixture volume per reaction, containing 0.16 pmol of forward primers, 0.16 pmol of reverse primers, and 10 ng of cDNA as template. All samples were performed in duplicate. Multiple negative controls lacking cDNA template were included in each assay. Relative gene expression analysis was carried out using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Gene expression levels were normalized against the expression level of ribosomal protein large P0 (*RPLP0*) housekeeping gene. BPV2 negative normal urinary bladder samples were used as the reference tissue for gene expression comparisons. Analysis of gene expression was not performed in all of the selected tumors due to sample depletion after extensive usage. Gene expression analysis was performed in 34 of the 38 tumors studied for *TP53*, *MDM2* and *EGFR* genes and in 36 tumors of the 38 for *CCND1* gene.

Table 16 – Sequences of primers used for gene expression analysis

Gene	Accession number	Sequence 5'-3'	Length (bp)
TP53	NM_174201.2	For: CCTCTGAGTCAGGAGACATTTTC	106
		Rev: GCTCGGAGGACAGAAGGTTATT	
MDM2	XM_003582211.3	For: GCAGTTAGTGAGACAGAAGAAAATTCAG	108
		Rev: GCAAGGCTTTCATCAAAGGAAA	
CCND1	NM_001046273.2	For: TCGTGGCCTCGAAGATGAAG	116
		Rev: GTTCACCAGGACCAGCTCCA	
EGFR	XM_592211.6	For: TGCCTTAGCCGTCTTATCCAA	122
		Rev: TGCAGAGGACAGGGTTGTTG	
RPLP0	NM_0010126	For: GCATTCCCGCTTCCTGG	109
		Rev: GCGCTTGTACCCATTGATGA	

5.3.4 Immunohistochemistry

Expression of *TP53*, *CCND1* and *EGFR* gene products, p53, Cyclin D1 and EGFR respectively, was assessed by immunohistochemistry in all samples, including the normal urinary bladders. The antibodies and the antigen retrieval methods used are shown in Table 17. Nuclear staining was considered positive for p53 and Cyclin D1, whereas membrane and/or cytoplasm staining was considered positive for EGFR. A 20% cutoff value was accepted as positive staining for p53 and Cyclin D1, similar to the previous studies of our group, and a 5% cutoff value was accepted as positive staining for EGFR. Bovine urothelial carcinomas with known p53 and Cyclin D1 positive immunohistochemical staining from previous studies were used as positive controls of the expression of those proteins. Additionally, normal bovine skin was used as a positive control for the expression of EGFR. Negative controls for immunohistochemistry were processed identically to the test slides, but the primary antibody was omitted. Due to tissue loss during the antigen retrieval process it was not possible to assess the expression of p53 in three samples and of EGFR in five. It was also not possible to confirm the expression of Cyclin D1 in 15 tumor samples due to tissue loss during antigen retrieval or to excessive background staining. It was not possible to perform immunohistochemical studies in one urothelial papilloma as insufficient paraffin-embedded material was available.

Table 17 – Antibodies and technical details used for immunohistochemistry

Antigen	Clone (Source)	Retrieval	Time/Dilution	Detection System (Source)
p53	DO7 (DakoCytomation, Carpinteria, CA)	Microwave, 600 Watts, 15 minutes in EDTA ph 9	Overnight/1:100	NovoLink Polymer Detection System (Leica Microsystems, Buffalo Grove, IL)
Cyclin D1	AM29 (Zymed Laboratories, CA)	Microwave, 600 Watts, 20 min in EDTA, pH 8	Overnight/1:100	NovoLink Polymer Detection System (Leica Microsystems, Buffalo Grove, IL)
EGFR	Ab2430 (Abcam, UK)	Microwave, 600 Watts, 20 minutes in citrate, ph 6	60 min/1:100	NovoLink Polymer Detection System (Leica Microsystems, Buffalo Grove, IL)

5.3.5 Statistical analysis

Statistical differences in the gene expression fold values found in BPV2 positive vs. BPV2 negative groups were compared using the student's *t*-test (SPSS version 23). The results were considered statistically significant when *p*-values were less than 0.05.

5.4 Results

5.4.1 Epithelial tumors

5.4.1.1 Gene expression

The relative gene expression results are presented in Table 18. *TP53* gene was found to be overexpressed in both urothelial papillomas (UP) and urothelial carcinomas (UC), without any statistically significant differences being found within the same group when comparing BPV2 positive and negative lesions (Table 18).

MDM2 gene expression profile in UP and UC groups had the largest variability of the genes studied, with a wide distribution of gene fold values within the same group. No statistical differences between BPV2 status groups were found (Table 18).

CCND1 gene was also found to be overexpressed in both UP and UC, with a larger variability seen on the UC. However, no statistical differences were found between BPV2 status groups (Table 18).

EGFR gene was expressed differently within the UP groups, being slightly overexpressed in BPV2 positive UP and clearly overexpressed in BPV2 negative UP ($p = 0.32$). The same pattern of expression was observed in the UC groups where BPV2 positive UC had lower EGFR gene expression levels than BPV2 negative UC ($p = 0.029$) (Table 18).

5.4.1.2 Immunohistochemistry

p53 protein nuclear accumulation was confirmed in 5 out 6 UP (Figure 13 a) and in 11 out of 13 UC (Figure 13 b). Variability in the intensity of staining was found.

Cyclin D1 overexpression was found in 2 out of 3 UP (Figure 13 c) and 7 out of 8 UC (Figure 13 d). As was seen with in p53 detection, variability in the intensity of the nuclear staining was observed.

All UP (Figure 13 e) and UC expressed the EGFR protein with some variability in the number of positive cells and in the staining intensity within the same tumor (Figure 13 f).

5.4.2 Endothelial tumors

5.4.2.1 Gene expression

The average values of *TP53* gene expression were increased in hemangiomas (Hg) and hemangiosarcomas (HgS), but a large variability was also present with the standard error of the mean being very close to the mean value in some groups (Table 18). No statistical differences were found between BPV2 positive and negative groups for either Hg or HgS. Overexpression of the *MDM2* gene was consistently increased in Hg by about 6-7 folds on both BPV2 status groups. On the other hand BPV2 positive HgS showed a very high expression variability contrasting with the BPV2 negative HgS. No statistical differences were detected either in Hg or HgS groups (Table 18).

CCND1 expression was slightly increased in BPV2 positive Hg, but the marked increase of gene expression observed in BPV2 negative Hg was paired with a large heterogeneity of values within the group. No statistical differences between groups were found. Regarding the HgS the increase of *CCND1* expression on both groups was minor but without statistical significance (Table 18).

Similar to the epithelial tumors, BPV2 positive Hg presented lower levels of *EGFR* gene expression when compared with BPV2 negative Hg ($p = 0.005$). The same conclusion could not be drawn from the HgS groups due to the small sample size (Table 18).

5.4.2.2 Immunohistochemistry

Although *TP53* gene expression levels were increased within this group of tumors, p53 nuclear accumulation was only seen in one out seven Hg (Figure 13 g) and was not observed in any of the eight HgS tested (Figure 13 h).

Cyclin D1 overexpression was confirmed in six out of six Hg (Figure 13 i) and in five out of five Hgs (Figure 13 j).

Five out six Hg (Figure 13 k) expressed EGFR protein, seen in the cytoplasm of the endothelial cells, and all seven HgS tested were EGFR positive (Figure 13 l).

Table 18 – Gene expression profile in BPV2 positive versus BPV2 negative urinary bladder tumors

Urinary Bladder Tumors	**UP+/UP- (mean ± S.E.M.)**	***p* value**	**UC+/UC- (mean ± S.E.M.)**	***p* value**	**Hg+/Hg- (mean ± S.E.M.)**	***p* value**	**HgS+/HgS- (mean ± S.E.M.)**	***p* value**
Gene								
TP53	**2.9**±1.9 (n=4)/ **3.8**±1.3 (n=2)	n.s.	**4.2**±0.4 (n=5)/ **3.2**±1.6 (n=7)	n.s.	**2.4**±2.2 (n=5)/ **3.5**±2.1(n=3)	n.s.	**5.3**±3.2 (n=5)/ **2.5**±1.1(n=3)	n.s.
MDM2	**3.2**±3.9 (n=4)/ **25.9**±20.2 (n=3)	n.s.	**9.5**±11.9 (n=4)/ **12.1**±16.6 (n=7)	n.s.	**6.2**±3.8 (n=5)/ **6.9**±1.8 (n=4)	n.s.	**16.7**±26.8 (n=5)/ **3.6**±1.7 (n=2)	n.s.
CCND1	**1.9**±0.9 (n=4)/ **3.2**±0.1(n=2)	n.s.	**1.9**±1.4 (n=6)/ **1.8**±1.3 (n=7)	n.s.	**1.4**±0.9 (n=5)/ **21.7**±19.8 (n=4)	n.s.	**2.2**±1.8 (n=5)/ **1.6**±1.2 (n=3)	n.s.
EGFR	**1.5**±0.5 (n=4)/ **3.4**±0.3 (n=2)	**0.032**	**0.2**±0.2 (n=6)/ **1.6**±1.5 (n=6)	**0.029**	**0.08**±0.05 (n=5)/ **1.8**±0.9 (n=4)	**0.005**	**1.8**±0.7 (n=5)/ **3.1** (n=1)	n.d.

UP+ - BPV2 positive urothelial papilloma; UP- - BPV2 negative urothelial papilloma; UC+ - BPV2 positive urothelial carcinoma; UC- - BPV2 negative urothelial carcinoma; Hg+ - BPV2 positive hemangioma; Hg- - BPV2 negative hemangioma, HgS+ - BPV2 positive hemangiosarcoma; HgS- - BPV2 negative hemangiosarcoma; S.E.M – standard error of the mean; n.s. – not significant; n.d. – not determined.

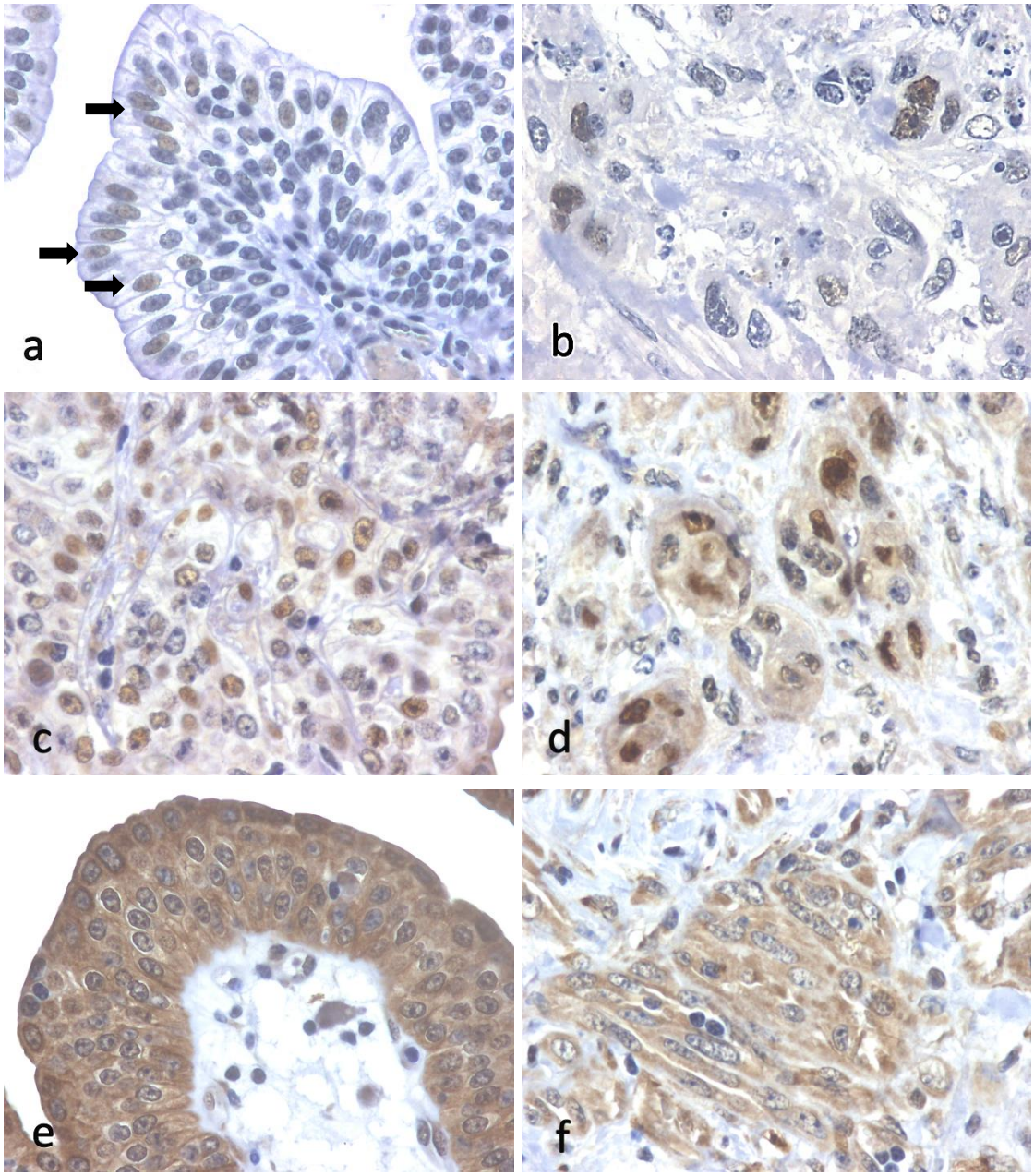

Figure 13 – Immunohistochemical detection of cell cycle regulators p53, Cyclin D1 and of the growth factor receptor EGFR in bovine urinary bladder neoplasms, positive for BPV2.

a – Urothelial papilloma with multiple nuclei (arrows) displaying weak p53 expression. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 200x.

b – Urothelial carcinoma infiltrating the lamina propria where several neoplastic cells present p53 expression. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

c – Urothelial papilloma with strong Cyclin D1 expression. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 200x.

d – Urothelial carcinoma invading the muscle layer with almost all of the neoplastic cells expressing Cyclin D1. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

e – Urothelial papilloma displaying strong cytoplasmic EGFR immunostaining. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 200x.

f – Urothelial carcinoma invading the muscle layer with diffuse cytoplasmic EGFR immunostaining of the neoplastic cells. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

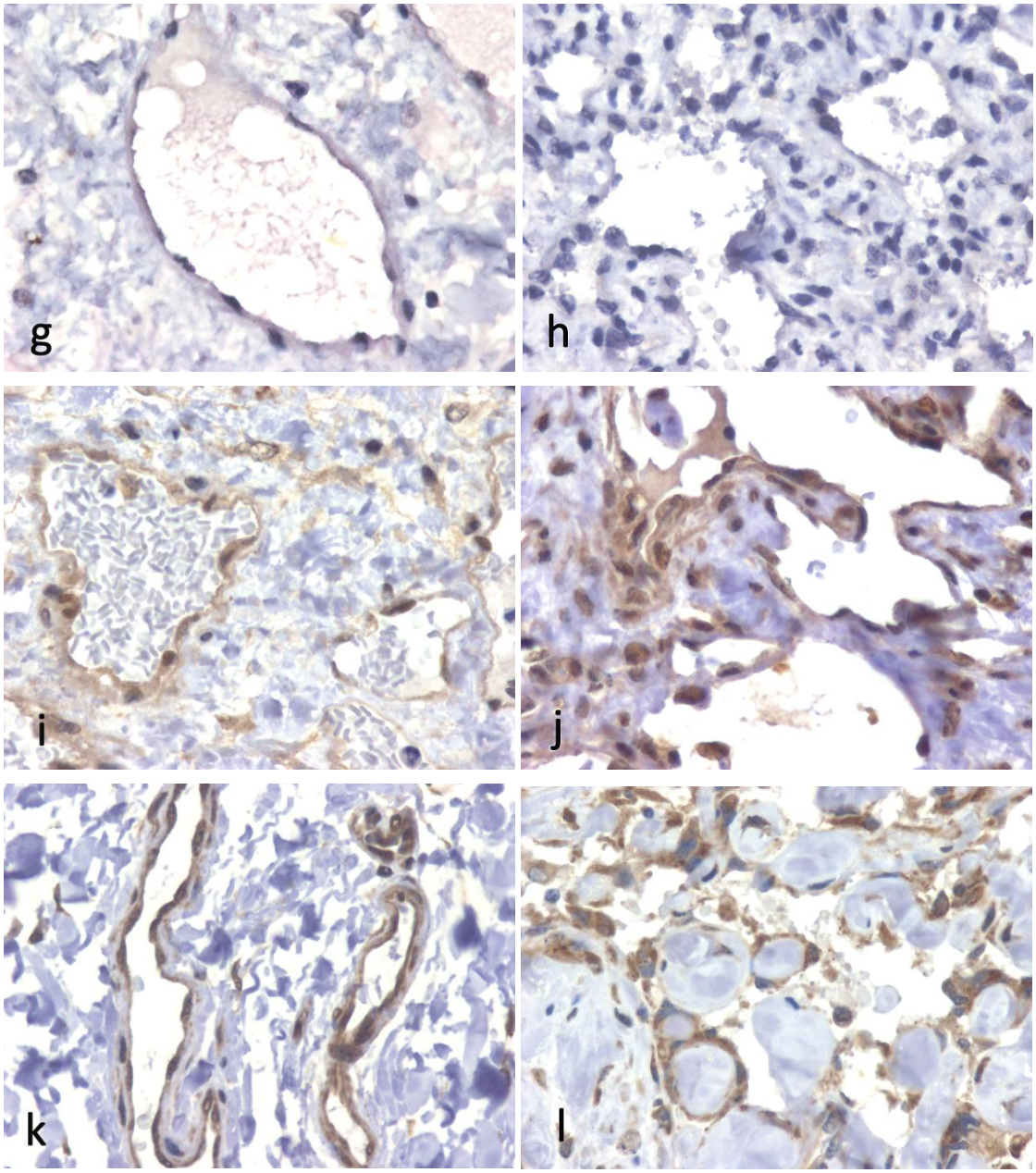

Figure 13 (continued)

g – Hemangioma negative for the expression of p53. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

h – Hemangiosarcoma negative for the expression of p53. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

i – Hemangioma with most of the neoplastic cells strongly positive for Cyclin D1. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

j – Hemangiosarcoma displaying a high number of nuclei strongly positive for Cyclin D1. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

k – Hemangioma with strong cytoplasmic EGFR expression in all of the endothelial cells. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

l – Hemangiosarcoma showing cytoplasmic EGFR expression in most of the endothelial neoplastic cells. NovoLink-peroxidase and Mayer's hematoxylin counterstain, 400x.

5.5 Discussion

The present work reports the gene expression patterns of cell cycle regulators as well as of *EGFR* in different groups of BPV2 positive and negative, epithelial and endothelial urinary bladder tumors of cattle suffering from BEH, in order to assess the possible role of this oncogenic virus in the molecular changes taking place in these lesions.

In the majority of the tumors studied, benign and malignant and regardless of the BPV2 status, *TP53* gene was overexpressed, and no statistical differences in gene expression levels between BPV2 status groups were found. *TP53* tumor suppressor gene, also known as the guardian of the genome, is responsible for cell cycle arrest in the presence of stress stimuli such as DNA damage, oncogene activation or hypoxia (Vousden and Lu, 2002). The immunohistochemical study confirmed the nuclear accumulation of p53 protein in most of the epithelial tumors, but not in the endothelial ones. These results suggest that *TP53* gene is frequently mutated in epithelial tumors but the same may not occur in endothelial tumors. Mutations of this gene are found in almost every type of human cancers at rates that can reach 50% (Olivier et al., 2010), including in urinary bladder tumors (Goebell and Knowles, 2010). Most of *TP53* mutations are missense, leading to the substitution of a single amino acid and to the expression of a very stable and inactive p53 protein in the nucleus. However in some cases frameshift or nonsense mutations can occur resulting in the loss of p53 expression (Muller and Vousden, 2014). This could be the case for the endothelial tumors studied in which p53 overexpression was not detected. Other studies on epithelial and vascular tumors of the urinary bladder of BEH-affected cattle have reported that p53 overexpression was associated with muscle-invasive urothelial carcinomas and muscle invasive hemangiosarcomas (Carvalho et al., 2009; Cota et al., 2014). However the number of tumors analyzed on those studies was much higher than of the present report, certainly affected the results obtained.

MDM2 gene expression levels were the most variable of all the genes studied. Although mean values of expression were above normal in all sample groups, the range of values found does not allow the statement of an unequivocal conclusion. When comparing BPV2 status groups no statistical differences were found. Furthermore, without the immunohistochemical evaluation these results could not be further confirmed. *MDM2* transcription is upregulated by p53, and MDM2 acts as a negative regulator of p53, leading to its destruction (Slaton et al., 2001). Overexpression of MDM2 is present in up to 30% of cases of human urinary non-invasive tumors, and gene amplification has been

found in 4% of muscle invasive bladder tumors (Goebell and Knowles, 2010). It is proposed that MDM2 counteracts the angiosuppressive action of p53 and is correlated with increased levels of vascular endothelial growth factor (VEGF), as was reported in angiosarcomas where MDM2 expression was clearly higher in comparison with the control tissues (Zietz et al., 1998). In the absence of mutated *TP53* gene, overexpression of *MDM2* could be an important step in the development of endothelial tumors of the urinary bladders of cattle. Further studies are necessary to confirm if the p53-MDM2 regulatory pathway is altered in BEH-affected cows.

The cell cycle promoter *CCND1* gene was found to be slightly overexpressed in both benign and malignant tumors of epithelial and endothelial origin, with no association with the presence of BPV2 DNA. The BPV2 negative Hg group demonstrated to be very heterogeneous regarding *CCND1* expression. Increased levels of *CCND1* mRNA were also reported in cases of human non muscle invasive bladder cancer (Kopparapu et al., 2013). There are not many studies concerning expression of this gene in vascular tumors either in man or in domestic animals. The overexpression of *CCND1* is mainly associated with gene amplification or with chromosomal rearrangements (Baffa et al., 2006) which can lead to protein overexpression. Cyclin D1 overexpression was identified in the majority of the epithelial tumors and in all of the endothelial ones. These are higher positivity rates than previous reports on Cyclin D1 expression in urinary bladder tumors of cattle (Carvalho et al., 2009; Cota et al., 2014) although the smaller sample size of present study could have influenced in the results. Genetic alterations that lead to Cyclin D1 overexpression are considered to be an early event in urinary bladder carcinogenesis (Baffa et al., 2006). Therefore it is expected that even low grade lesions can harbor such genetic alterations leading to Cyclin D1 overexpression.

EGFR gene expression levels differed between BPV2 status groups in the case of UP ($p = 0.032$), UC ($p = 0.029$) and Hg ($p = 0.005$), in which the BPV2 positive tumors expressed lower levels of this growth factor receptor gene when compared with the BPV2 negative corresponding tumors. EGFR protein expression was detected in all tumors studied, except for one Hg. Previously a similar result was described in tumor endothelial cells in mice (Amin et al., 2006). The expression of *c-ErbB2* gene, which belongs to the ErbB family of tyrosine kinases as EGFR, and that encodes the Her-2 receptor was assessed by immunohistochemistry in 17 urinary bladder inflammatory, non-neoplastic and neoplastic lesions of cattle and buffalos (Kumar et al., 2014). In that study, where the sample size was small and the lesion heterogeneity was large, only 35% of the lesions

were c-ErbB2 positive. Furthermore the authors reported that only epithelial cells expressed c-ErbB2 (Kumar et al., 2014). A recent study has also reported the expression of *EGFR* gene and protein in canine urothelial tumors which was higher when compared with normal urinary bladder samples (Hanazono et al., 2015). EGFR overexpression has already been reported in several carcinomas in man, and is associated with tumor progression and metastasis (Normanno et al., 2006). To the best of the author's knowledge this is the first report on bovine endothelial tumor cells expressing EGFR. EGFR is required for the induction epidermal hyperplasia and tumor formation in HPV-associated tumors, in which EGFR levels are increase in response to the E5 protein (reviewed in DiMaio and Petti, 2013). In the present study the opposite was observed, with BPV2 positive tumors expressing lower levels of *EGFR* gene. Similarly, less intense immunohistochemical staining was observed in some of the BPV2 positive tumors comparing with the BPV2 negative ones. Furthermore BPV2 DNA in the tumors used in this study was present at very low levels, and no transcriptional activity was detected (Cota et al., 2015). These results were unexpected as none of the other gene expression profiles were associated with BPV2 infection. This finding is quite new in the BEH pathology scenario and further studies are required to better understand the possible interaction between BPV2 and *EGFR* in urinary bladder tumors of cattle.

In BEH there is a considerable histological variability of the lesions present in the urinary bladders of the affected animals (Carvalho et al., 2006; Pamukcu et al., 1976; Peixoto et al., 2003). The number, nature and severity of the lesions found in BEH cases is thought to be related with the amount and duration of exposure to the bracken fern toxic components (Shahin et al., 1998b). The tumors studied in the present report were selected mainly by their BPV2 status and not for their histopathological features namely tumor grade or invasion stage, though tumor characteristics (non-invasive vs. invasive/well differentiated vs. poorly differentiated) were taken into account and samples with different pathological features were included in each group. Lesions of the same histological origin may have the same starting point, undergoing the same carcinogenic pathway, but the accumulation of different genomic alterations may lead to lesions with diverse degrees of differentiation and invasive capability which can be present within the same urinary bladder. Similarly, the development of tumors in BEH was suggested to be a continuous process comprising successive stages of the same oncological process (Carvalho et al., 2006). Future studies with pre-malignant, low grade and high grade

lesions could point out which molecular events are occurring and which pathways lead to tumor progression.

Since the studies of Campo et. al (1992) BEH has been looked upon as a disease with more than one player, resulting from the interaction of the toxins present in bracken fern and BPV. A similar scenario is seen in human cervical cancer where environmental cofactors, such as tobacco smoke and its related carcinogens, are proposed to be involved in the HPV related carcinogenesis process (Castellsague and Munoz, 2003). Some studies have reported the effects of BPV and its oncoproteins in urinary bladder tumors (Borzacchiello et al., 2007, 2006; Roperto et al., 2014) suggesting a major role of BPV in BEH associated carcinogenesis. In a previous work on BPV2 quantification in urinary bladder lesions of cattle affected by BEH no statistical association between BPV2 and histological type of lesion was found, there was a high prevalence of BPV2 in both healthy and BEH-affected animals and no BPV transcriptional activity (Cota et al., 2015). In fact, the extent of the participation of BPV in naturally occurring urinary bladder tumors is still far from being fully understood.

In conclusion, the expression of the cell cycle controlling genes *TP53*, *MDM2* and *CCND1* is altered in urinary bladder tumors of BEH affected cattle, the cause of this alteration being possibly associated with gene mutations and/or gene amplifications that should be further studied. The BPV2 status of the tumors did not affect the expression values of these genes suggesting that the effect of this oncogenic virus does not alter these pathways. Contrarily, BPV2 presence seemed to be associated with lower gene expression values of *EGFR*, which recommends that future work should be designed to address this possible interaction.

Chapter VI – Evaluation of the effect of BPV2 E5, E6 and E7 oncoproteins in bovine cells – a preliminary study

6.1 Abstract

BPVs are small DNA viruses that can contribute to the development of malignant tumors in cattle such as urinary bladder tumors. This preliminary study aimed to evaluate the effect of the BPV2's oncoproteins present in BEH-associated tumors in an *in vitro* model using MDBK bovine kidney cells. Replication-defective retroviruses encoding E5, E6 and E7 genes were produced and used to generate MDBK cell lines that could stably express these oncogenes. Although cell transformation was not achieved with any of the BPV2 oncoproteins studied, changes in the cellular growth rate and in gene expression levels were observed, suggesting that these proteins may function in other ways that are still not fully understood.

6.2 Introduction

BPVs are small DNA viruses that infect the epithelia of their natural hosts and give rise to epithelial proliferative benign lesions known as papillomas (de Villiers et al., 2004). Under normal conditions BPVs produce benign cutaneous and/or mucocutaneous epithelial lesions in cattle which generally regress, but when associated with environmental co-factors those benign lesions can progress to form malignant tumors, specifically in the upper alimentary canal and the urinary bladder (Campo, 2006).

The BPVs double-stranded circular genomes are approximately 8 kb in size and have three different regions: a non-coding long control region (LCR), an early region generally with 5 to 6 open reading frames (ORFs) encoding non-structural proteins, and a late region with 2 ORFs encoding the capsid proteins (Nasir and Campo, 2008; Rector and Van Ranst, 2013). Of the non-structural proteins encoded in the early region three, E5, E6 and E7, are of particular importance due to their transforming activity and for this reason are designated oncoproteins.

The BPV1 E5 oncoprotein, with merely 44 amino acids, is the major player in the BPV associated cellular transformation process *in vitro* through physically interacting with and activating the platelet derived growth factor β receptor (PDGFR- β) (DiMaio et al., 1986; Petti and DiMaio, 1994; Petti et al. 1991). Since the E5 protein homodimerizes, when it binds to the PDGFR-β it causes dimerization, which in turn activates the receptor stimulating mitogenic signaling (Petti and DiMaio, 1994; DiMaio and Petti, 2013). The BPV2 E5 oncoprotein, which has an identical amino acid sequence as that of BPV1 E5, has been suggested to have a similar biological role in naturally occurring urinary bladder tumors of cattle (Borzacchiello et al., 2006). Other E5 activities have been described, such as down-regulation of the major histocompatibility complex (MHC) class I on the cell surface and an interaction with the 16K vacuolar-ATPase subunit, revealing the many functions of such a small protein (reviewed in DiMaio and Petti, 2013).

The BPV1 and BPV2 E6 proteins share 84% amino acid sequence identity, are 137 amino acids long, and like HPV16 E6, are characterized by having Cys-X-X-Cys zinc binding motifs (Tong and Howley, 1997; Tomita et al., 2007), which engage interactions with cellular proteins (Zanier et al., 2013). The BPV1 E6 binds to paxilin, a focal adhesion molecule, disrupting the actin cytoskeleton, and this interaction correlated with the ability of E6 to transform cells (Tong and Howley, 1997). BPV1 E6 was shown to block the interaction of paxilin with vinculin and focal adhesion kinase (Tong et al., 1997). Other

in vitro studies reported further that BPV1 E6 interact with the clathrin adapter AP1 (Tong et al., 1998), the transcriptional coactivator CBP/p300 leading to the inhibition of the tumor suppression protein p53 (Zimmermann et al., 2000), and more recently to the mastermind-like protein 1 (MAML1) affecting the NOTCH pathway (Brimer et al., 2012). Both BPV1 and BPV2 E6 oncoproteins still have not been fully studied in naturally occurring tumors, despite the numerous *in vitro* studies indicating the oncogenic potential of BPV1 E6 (Corteggio et al., 2013).

The BPV1 E7 and BPV2 E7 proteins are 94% identical in their amino acid sequence. Each protein is 127 amino acids in length and has a zinc binding motif like E6 (Tomita et al., 2007). However, a major difference between these E7s and those encoded by other PVs genomes, such as BPV4 or the HPVs, because they do not bind to the retinoblastoma tumor suppressor (pRB) family of proteins due to the lack of the Leu-X-Cys-X-Glu motif (Münger et al., 1989; Pennie et al., 1993;Tomita et al., 2007). In *in vitro* studies BPV1 E7 alone did not have an evident oncogenic effect in C127 cells, but when it was expressed together with E5 or E6 it increased their transforming capacities (Bohl et al., 2001). DeMasi et al, (2005) found that BPV1 E7 interacts with a 600 kDa cellular protein, p600, which is involved in membrane morphogenesis and cell survival (Nakatani et al., 2005). BPV1 E7 was also found to inhibit anoikis, an apoptosis mechanism by which anchorage-dependent cells die when not attached to the extracellular matrix, and this characteristic partially correlated with the E7-p600 interaction (DeMasi et al., 2007). The BPV2 E7 oncoprotein was found in a series of urinary bladder lesions of BEH-affected cattle, but the exact role on the development of neoplastic lesions is still unknown (Borzacchiello et al., 2007; Borzacchiello et al., 2009).

Molecular changes in the genes that control the cell cycle are important steps in the carcinogenesis of the urothelial tumors of the urinary bladder (Yurakh et al., 2006). TP53 tumor suppressor gene deletion or mutations are found in 70% of muscle invasive bladder cancer (MIBC) cases. The amplification and/or overexpression of *CCND1* and *MDM2* oncogenes account for 10-20% and 4%, respectively, of the genetic alterations found in MIBC in men (Goebell and Knowles, 2010). In urothelial bladder tumors of cattle *TP53* mutations and *CCND1* overexpression have been reported (Cota et al., 2014).

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of tyrosine kinases and its abnormal expression has been reported in carcinomas arising from several organs including the urinary bladder (Yarden and Sliwkowski, 2001). This growth factor receptor can play a crucial role in epithelial neoplasms enhancing tumor growth, invasion

and metastasis (Normanno et al., 2006). Thus it is considered as an important therapeutic target.

The objective of this preliminary *in vitro* study was to evaluate the effect of expressing the BPV2 E5, E6 and E7 proteins in bovine epithelial cells. Most previous studies examining the role of the BPV early genes in *in vitro* transformation were performed using murine fibroblasts, which do not accurately represent the epithelial tumors found in BEH. Therefore, in order to clarify the role of BPV2 in the BPV2 in the BEH-associated carcinogenic process, we used replication-defective recombinant retroviruses to introduce and stably express the BPV2 E5, E6 and E7 genes individually in bovine kidney epithelial cells. We hypothesized that at least one of these genes would affect changes in cell behavior or gene expression that might contribute to transformation. Furthermore, we aimed to correlate changes in cellular gene expression induced by these BPV2 genes with the gene expression differences seen in the urinary bladder tumors associated with BEH. The work developed here can serve as a framework for future studies unveiling the key events induced by BPV2 during the *in vitro* and *in vivo* neoplastic transformation process.

6.3 Materials and methods

6.3.1 Cell lines

Madin-Darby Bovine Kidney (MDBK) cell line, kindly provided by Professor Miguel Fevereiro from Instituto Nacional de Investigação Agrária e Veterinária, I.P. (INIAV), was used to determine the effect of the BPV2's oncoproteins. The 293T Human embryonic kidney cell line, kindly provided by Professor Aida Esteves from the Instituto de Higiene e Medicina Tropical, was used as the replication-defective retrovirus packaging cell line.

6.3.2 Cell manipulation

All of the procedures used in the manipulation of the cell lines were performed under aseptic conditions in a vertical laminar flow hood. MDBK and 293T cell lines were grown in tissue culture flasks with Advanced MEM supplemented with L-glutamine Gibco® (2 mM), 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B

(0-.25 µg/ml) and stored in an cell incubator at 37ºC with 5% CO_2. The cells were routinely observed using an inverted microscope.

Cell line stocks were achieved through cryopreservation. Briefly, cells were grown to 80-90% confluency and were trypsinised using 0.05% Trypsin-EDTA. Advanced MEM was added after cell detachment and the cell suspension was centrifuged at 800g for 10 minutes. The cell pellet was re-suspended in FBS with 10% Dimetyl sulfoxide (DMSO) (Sigma-Aldrich) and divided in 1 ml aliquots per cryovial. The cryovials were stored in a -80ºC freezer for a 24 hour period and then transferred to a liquid nitrogen container for long term storage.

6.3.3 Plasmids

The retroviral expression vector plasmids used were the pBabepuro plasmid, encoding a puromycin resistance gene, and the recombinant pBabepuro E5 encoding the BPV1 E5 gene. The pVSV-G retroviral envelop plasmid and pCl-Eco retroviral packaging plasmids were also used for the production of retroviruses in 293T cells. All of these plasmids were kindly provided by Professor Daniel DiMaio from Yale University, New Haven, CT.

6.3.3.1 E6 and E7 Retroviral Plasmid construction

As BPV1 and BPV2 E5 oncoproteins are identical the recombinant pBabepuroE5 provided by Professor Daniel DiMaio was used. BPV2 E6 and E7 genes were amplified from BPV2 positive bovine cutaneous papillomas using the primers disclosed in Table 19. The amplicons obtained were cloned into a pGEM®-T-Easy Vector (Promega Corporation, Madison, USA) according to the manufacturer's instructions, and the recombinant plasmids were used to transform *Escherichia coli* Dh5α competent cells prepared by the calcium chloride method (Cohen et al., 1972). Plasmid DNA was purified using ZR Plasmid Miniprep™ Classic (Zymed Research, Irvine, USA) and was sequenced to ensure that the inserts retained the identity with the E6 and E7 genes of BPV2. To clone the E6 and E7 genes in the pBabepuro retroviral plasmid directional cloning was performed using the *EcoRI* (1379-1384 bp) and the *SalI* (1397-1402 bp) restriction enzyme sites present in the plasmid multiple cloning site. New primers were designed, containing in the 5' end of the forward primer the *EcoRI* site, and a Kozac

sequence including the start codon of the gene. The reverse primer was designed to contain in the 5' end the *SalI* site followed by the stop codon of the gene (Table 19). The E6 and E7 genes were amplified by PCR using the pGEM® recombinant plasmids encoding the E6 and E7 genes as templates and the above mentioned primers with the restriction enzyme sites. The amplicons and the pBabepuro plasmid were hydrolyzed with Speedy *EcoRI* and Speedy *SalI* restriction enzymes (Nzytech, Portugal) and ligated using the Speedy T4 DNA ligase (Nzytech, Portugal) according to the manufacturer's instructions. The resulting recombinant retroviral plasmids were used to transform *Escherichia coli* Dh5α competent cells. These recombinant retroviral plasmids were purified using the ZR Plasmid Miniprep™-Classic (Zymed Research, Irvine, USA) and sequenced to confirm the directional cloning of BPV2's E6 and E7 genes.

Table 19 – Primers used for recombinant retroviral plasmids construction

Gene	Sequence 5'-3'
E6	For: AAGCTGCTGACAGACCCTGATTCC (65-88 bp)*
	Rev: GGTCCTGCAGGTGAATCATCCAAG (531-508 bp)*
E7	For: TAAGAGGACGCTGCTACGACTGCTG (449-473 bp)*
	Rev: GATATGAGCATCCCAATGCCGAATC (900-876 bp)*
E6p	For: G*GAATTC***ACCATGG**ATGGACCTGAAACCTTTGG
	Rev: ACGC*GTCGAC*CTATGGGTATTTGGACCTTGAACCATG
E7p	For: G*GAATTC***ACCATGG**ATGGTTCAAGGTCCAAATAC
	Rev: ACGC*GTCGAC*TTATCGTTTGCCATGACGCTCGCGAGA

*Nucleotides in the BPV2 genome (GenBank accession number M20219-1);

Primers sequences: E6p forward primer has one nucleotide before the *EcoRI* restriction site (italic) for an efficient hydrolysis, the Kozac sequence (bold), and the starting codon (underlined); E6p reverse primer has four nucleotides before the *SalI* restriction site (italic) for an efficient hydrolysis, and the stop codon (underlined); E7p forward primer has one nucleotide before the *EcoRI* restriction site (italic) for an efficient hydrolysis, the Kozac sequence (bold), and the starting codon (underlined); E7p reverse primer has four nucleotides before the *SalI* restriction site (italic) for an efficient hydrolysis, and the stop codon (underlined).

6.3.4 Production of replication-defective retroviruses

The calcium phosphate transfection method was applied for the production of replication-defective retroviruses. 293T cells were grown in 100 mm dishes with Advanced MEM supplemented with Penicillin/Streptomycin/Amphotericin in the above mentioned concentrations and 10% FBS to a 60-90% confluency. Five hundred µl of 2x HEBS were mixed with 4 µg of pVSV-G, 6 µg of pCL-Eco and 10 µg of the recombinant retroviral vector. Five hundred µl of 0.25 M $CaCl_2$ were then added to the mix while bubbling air with a 1 ml pipette and the precipitate was incubated for 20 minutes at room temperature. The mix was then added to the 293T cell dishes dropwise, which afterwards were placed in the cell incubator. After 48h, the virus-containing medium of the transfected 293T cell dishes was collected and filtered through a 0.45 µm syringe filter, and the flow through containing the recombinant retroviruses was stored at -80º until use.

6.3.5 MDBK cell infection and selection

The MDBK cells were grown in 60 mm dishes with Advanced MEM supplemented with Penicillin/Streptomycin/Amphotericin in the above mentioned concentrations and 10% FBS and were infected at low confluency (40-60%) with two ml of the recombinant retrovirus supplemented with polybrene (Sigma-Aldrich) at a final concentration of 2 µg/ml. After 24 hours, the infected MDBK cells were trypsinized and plated into 100 mm dishes with cell medium containing puromycin (Sigma-Aldrich) at a final concentration of 4 µg/ml. Non-infected (mock) MDBK cell dishes were used as negative controls.

6.3.6 RNA extraction and cDNA production

Total RNA was extracted from the transduced cells and from mock MDBK cells using the RNeasy® Mini kit (Qiagen, Germany), according to the manufacturer's protocol. The RNA concentration and purity was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA) and was stored at -80 ºC until use. One microgram of RNA from each sample was treated with 1 unit of DNase I, accordingly to the manufacturer's protocol (Invitrogen, USA), to eliminate any genomic DNA present,

and was subjected to reverse transcription using anchored Oligo dTs with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland).

6.3.7 BPV2 E5, E6 and E7 oncogene expression analysis

To confirm the transcription of the viral oncogenes in the MDBK transduced cells, quantitative PCR (qPCR) was performed using cellular cDNA as template. The primers (Table 20) were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and purchased from Stab Vida Genomics Lab Portugal. Detection of BPV2's E5, E6 and E7 mRNA was performed in a StepOnePlus™ Real-time PCR system (Applied Biosystems, Foster City, USA), using Luminaris Color HiGreen High ROX qPCR Master Mix (Life Technologies). All amplifications were performed in a 12.5 µl mixture volume per reaction, containing 0.16 pmol of forward primers, 0.16 pmol of reverse primers, and 10 ng of cDNA as template. The cycling conditions included an initial denaturation at 95ºC for 10 min followed by 45 cycles of a denaturation step at 90ºC for 15 seconds and annealing and extension steps at 60ºC for 1 minute, followed by a melting curve step. All reactions were performed in duplicate. Multiple negative controls lacking cDNA template were included in each assay.

Table 20 – Sequence of primers used for oncogene expression analysis

Gene	Accession number	Sequence 5'-3'	Length (bp)
E5	M20219.1	For: AATCTATGGTTTCTATTGTTCTTGGGACTA	100
		Rev: CAAAATGATCCCAGTATACCAAGAAAA	
E6	M20219.1	For: GCTGCTACTGTGGGGGAAAA	114
		Rev: AGCAGTCGTAGCAGCGTCCT	
E7	M20219.1	For: TCCTCTCCCGGAAAAGTGTG	124
		Rev: AGCCCAGCAAGGTCGTAGAG	

6.3.8 Quantitative gene expression analysis

Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and purchased from Stab Vida Genomics Lab Portugal (Table 21). The primers were designed in the exon-exon junction areas of the genes. Quantification of *TP53*, *CCND1*, *MDM2* and *EGFR* gene transcripts was performed using StepOnePlusTM Real-time PCR system (Applied Biosystems, USA) and the Luminaris Color HiGreen High ROX qPCR Master Mix (Life Technologies). All amplifications were performed in a 12.5 µl mixture volume per reaction, containing 0.16 pmol of forward primers, 0.16 pmol of reverse primers, and 10 ng of cDNA as template. The same cycling conditions mentioned above were used. All reactions were performed in duplicate. Multiple negative controls lacking cDNA template were included in each assay. Relative gene expression analysis was carried out using the $2^{-\Delta\Delta C}{}_t$ method (Livak and Schmittgen, 2001). Gene expression levels were normalized against the expression level of the ribosomal protein large P0 (*RPLP0*) housekeeping gene.

Table 21 – Sequence of primers used for quantitative gene expression analysis

Gene	Accession number	Sequence 5'-3'	Length (bp)
TP53	NM_174201.2	For: CCTCTGAGTCAGGAGACATTTTC	106
		Rev: GCTCGGAGGACAGAAGGTTATT	
CCND1	NM_001046273.2	For: TCGTGGCCTCGAAGATGAAG	116
		Rev: GTTCACCAGGACCAGCTCCA	
MDM2	XM_003582211.3	For: GCAGTTAGTGAGACAGAAGAAAATTCAG	108
		Rev: GCAAGGCTTTCATCAAAGGAAA	
EGFR	XM_592211.6	For: TGCCTTAGCCGTCTTATCCAA	122
		Rev: TGCAGAGGACAGGGTTGTTG	
RPLP0	NM_0010126	For: GCATTCCCGCTTCCTGG	109
		Rev: GCGCTTGTACCCATTGATGA	

6.3.9 Cell growth rate curves

Multiple 60 mm tissue culture dishes were plated with 20.000 cells of MDBK, MDBKE5, MDBKE6 and MDBKE7 cell lines, at 0 hours, and the number of viable cells was counted at 24, 48, 72 and 96 hours post-plating. For each time-point three 60 mm dishes were counted. Mock MDBK cells were used as controls. After trypsinisation, a sample of the supernatant was mixed with trypan blue dye, at a 1:4 dilution, and loaded in the Neubauer chamber. The cells were counted using a microscope at a 100x magnification.

6.3.10 Statistical analysis

The data collected from the cell counting at the different time points to produce the cell growth curve was analyzed One-Way ANOVA followed by Tukey post-hoc test. The results were considered statistically significant when P values were less than 0.05. Statistical analysis was performed using SPSS version 23.

6.4 Results

6.4.1 Expression of E5, E6 and E7 mRNA in MDBK cells

Expression of E5, E6 or E7 transcripts in the respective transduced cell line was assessed by Real-Time PCR. Expression of these genes was detected, but at lower levels than the reference gene RPLP0 in all cell lines (Table 22).

Table 22 – Detection of E5, E6 and E7 oncogene expression in MDBK transduced cell lines

Cell line	RPLP0 C_T	E5 C_T	E6 C_T	E7 C_T
MDBKE5	22,13	40,25	-	-
MDBKE6	22,22	-	30,88	-
MDBKE7	22,16	-	-	26,27

C_T – threshold cycle.

6.4.2 Morphologic analysis

The expression of E5, E6 or E7 did not affect the cell morphology when compared with the non-transduced cells (Figure 14). The transduced cells maintained the variable cell morphology seen in the MDBK control cells, ranging from fusiform to hexagonal shapes at subconfluent densities. The nucleus/cytoplasm ratio, and the presence of multiple visible nucleoli, was maintained. The formation of cytoplasmic projections commonly seen in the MDBK cells was observed in all three transduced lines. The number of mitoses seen seemed to be higher in MDBKE5 and MDBKE6 when compared with the MDBKE7 cell line.

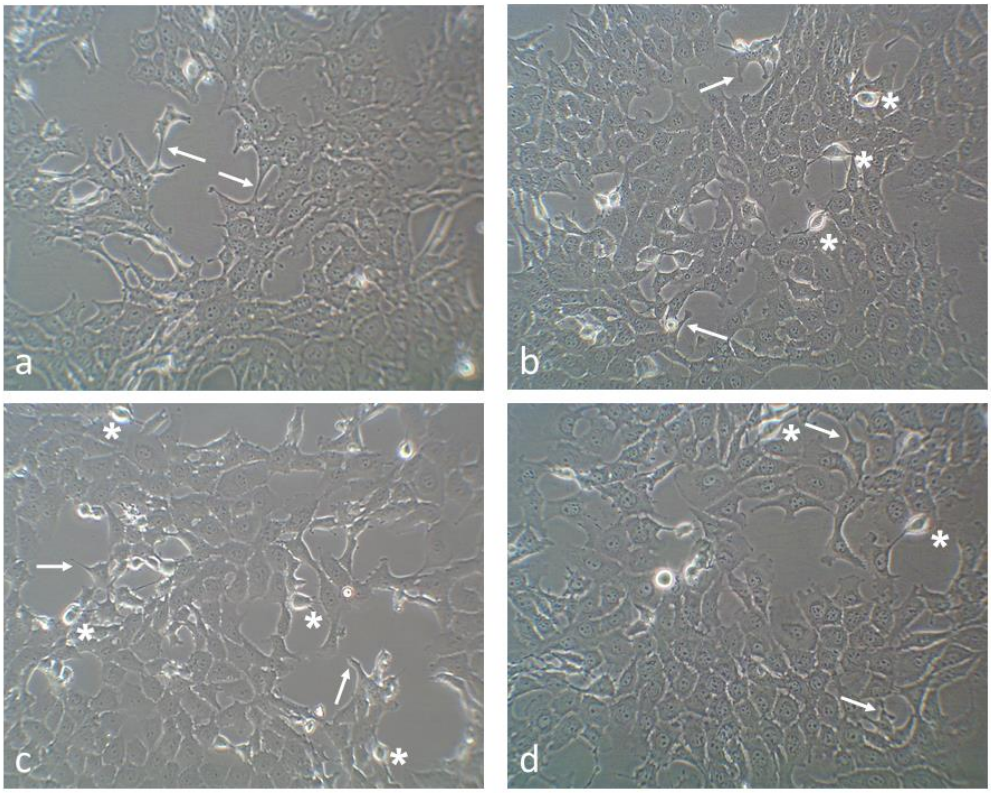

Figure 14 – Comparison of the transduced cell morphology (amplification 200x).
The arrows draw attention to the cytoplasmic projections, the asterisks point out the cells undergoing mitosis. a - Control MDBK cells; b – MDBKE5 cells; c – MDBKE6; d – MDBKE7.

6.4.3 Cell growth rate

Distinct growth patterns of the MDBKE5, MDBKE6 and MDBKE7 cells were observed (Figure 15). Most notably, MDBKE7 cells grew at a slower rate than the control cells, with the cell numbers being significantly lower ($p < 0.05$) at 48h and 72h post seeding. MDBKE5 and MDBKE6 displayed a similar growth pattern to each other and appeared to grow at a slightly faster rate than the control MDBK cells.

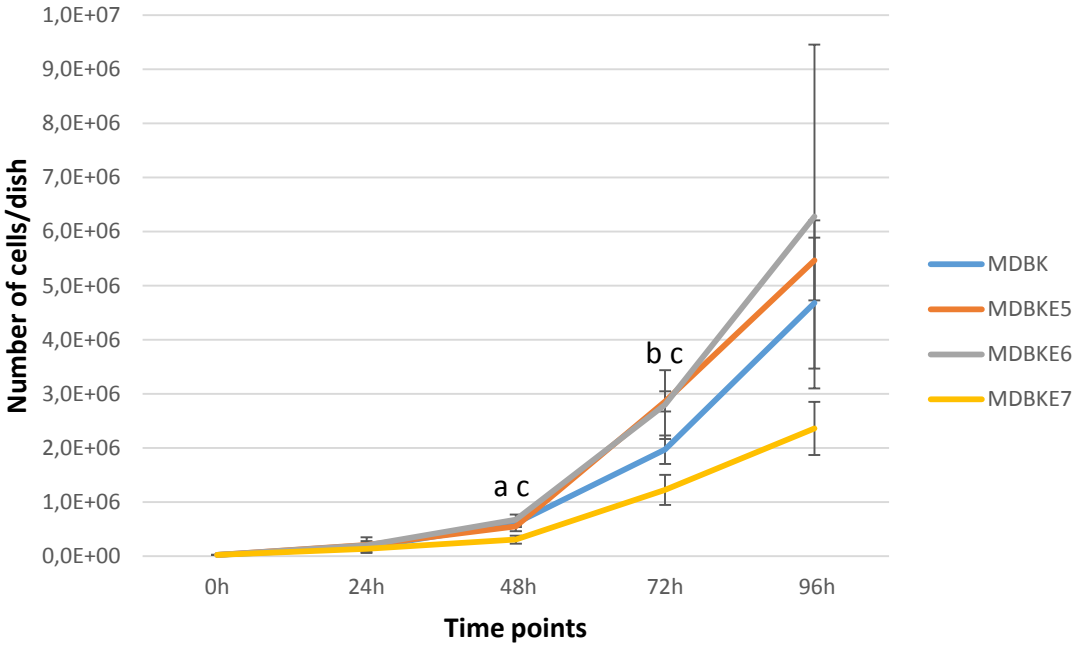

Figure 15 – Growth curve of MDBK cells expressing BPV2's putative oncoproteins. Values represent the average number of cells at different time points. Error bars represent standard error of the mean. Letters identify statistically significant different values ($p<0.05$) between cell lines at the specified time point: a - MDBK ≠ MDBKE7; b – MDBKE5 ≠ MDBKE7; c – MDBKE6 ≠ MDBKE7.

6.4.4 Gene expression alterations

When compared with the MDBK control cells, all three transduced cell lines showed over-expression of the *TP53* gene, with a fold increase over the control cells of 2.2, 4.2 and 11.8 times in the MDBKE6, MDBKE5 and MDBKE7 cells, respectively (Figure 16). The *EGFR* gene was also over-expressed in all three cell lines, with its expression being increased by 2, 2.5 and over 5 folds in the MDBKE6, MDBKE5 and MDBKE7

cells, respectively. On the other hand, the *CCND1* gene was under-expressed in all three cell lines. *MDM2* gene expression was increased in the MDBKE5 and MDBKE6 cells but not in the MDBKE7.

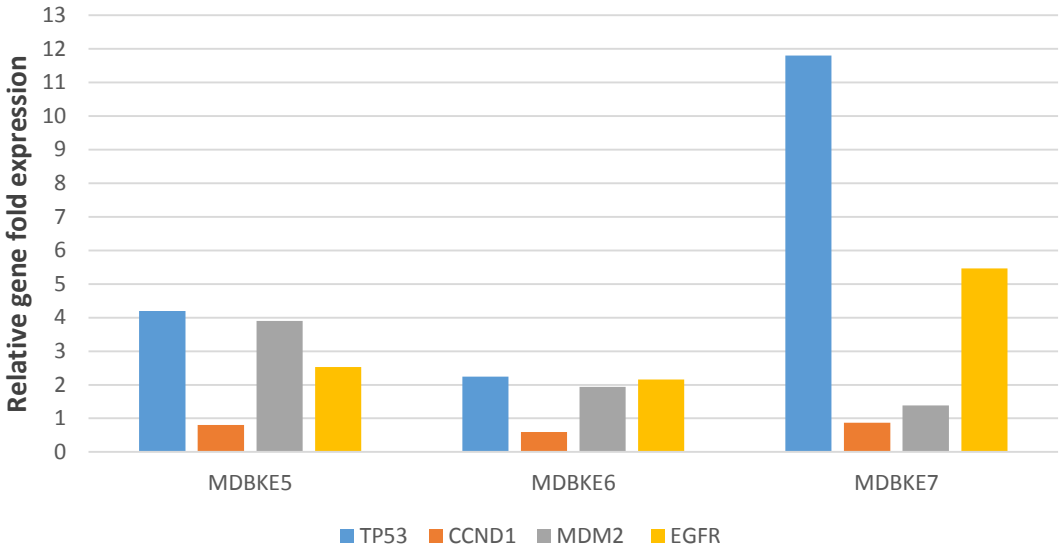

Figure 16 – Relative gene expression in the transduced MDBK cells compared with control cells. Normal gene expression was considered to be 1 fold.

6.5 Discussion

BPV1 has been used as a model to study the process of papillomavirus-induced cellular transformation (DeMasi et al., 2005). These studies were performed mainly in mammalian cell lines other than bovine. In contrast, BPV2, which also has the capability to transform cells (Dvoretzky et al., 1980; Campo and Spandidos 1983), and has been associated with naturally occurring neoplastic disease (Campo et al., 1992), has not been studied extensively. Therefore, we set out to investigate the role of the putative BPV2 oncogenes, E5, E6 and E7, in the transformation process of cells derived from the natural host of this virus. Specifically, we examined the effects of persistently expressing these genes individually in the bovine epithelial cell line MDBK using replicative-defective retroviruses.

In the present study neither BPV2 E5, E6 nor E7 induced visible morphological changes in the MDBK cells, even though the transcription of these genes was confirmed. This differs from studies of the corresponding BPV1 oncogenes in various fibroblast lines. It is well established that BPV1 E5 can induce the morphological transformation of human,

murine and equine fibroblasts (Bergman et al., 1988; Petti and Ray, 2000; Yuan et al., 2011), an activity which requires the PDGFR- β. Presumably BPV2 E5 should have the same activity in fibroblasts since its amino acid sequence is identical to that of BPV1 E5. When expressed in murine C127 fibroblasts BPV1 E6 was also shown to induce marked changes in the cell morphology, which were related to the disruption of the actin cytoskeleton structure caused by this oncoprotein (Tong and Howley, 1997). Again, this altered phenotype was not observed in the MDBK cells harboring and expressing the BPV2 E6 gene. In a different study, murine fibroblasts expressing BPV1 E7 showed no morphological differences compared to control cells (Fan et al. 2003). Of the three BPV1 oncoproteins, E7 was the only one that by itself was not capable of inducing great morphological or behavioral changes, but enhanced the caused by E5 and E6 (DeMasi et al., 2007). This synergy feature of BPV E7 was not assessed in this preliminary study in MDBK cells. However, further studies on the combined cytopathic effect of BPV2's putative oncogenes in MDBK cells as well as their effect in primary bovine fibroblasts are being prepared.

The MDBKE5 and MDBKE6 cells had an apparent faster growth rate between the 48 and the 96 hours post seeding, but this feature did not have any statistical significance in the present study. Petti and Ray, 2000, reported that human fibroblasts expressing E5 reached a 2-3 fold higher saturation density when compared with the control cells. This effect, like the other transforming phenotypes induced by BPV1 E5 in these cells, was likely due to the ability of E5 to bind and activate the PDGFR-β, which is expressed abundantly in fibroblasts but absent in MDBK cells. Any increase in growth induced by BPV2 E5 in MDBK cells could be due to the ability of E5 to bind to a different target in epithelial cells. This is consistent with the finding that BPV1 E5 could tumorigenically transform murine keratinocytes, which also do not express the PDGFR- β (Leptak et al., 1991).

In the present study the MDBK cells expressing BPV2 E7 surprisingly displayed a significantly slower growth rate than the control cells. The slowed growth rate of MDBKE7 cells correlated with a substantial increase in *TP53* gene expression. In fact, these cells displayed the highest level of *TP53* gene expression of all of the cell lines established. These results suggest that BPV2 E7 might induce changes in the cell that increase p53 transcription, which in turn decreases the rate of cell division. In contrast, Fan et al., in 2003 reported that BPV1 E7 induced DNA synthesis and drives quiescent cells in to the S phase in murine fibroblasts, features which were associated with the activation of c-Myc gene. It is possible that the BPV1 and BPV2 E7 proteins behave

differently from each other, or their function in epithelial cells is very different than that in fibroblasts. To the best of our knowledge, this effect of slowing cell proliferation and increasing p53 expression has not been reported for a papillomavirus E7 gene. The implications of these results for BPV2-induced cell transformation and virus replication should be pursued further.

Despite the absence of morphological transformation, different gene expression profiles were detected in cells expressing BPV2 E5, E6 or E7 when compared to the control cells. Although these preliminary results do not seem to be similar to those found in BEH-associated urinary bladder tumors (Cota et al., paper in preparation). The most striking difference concerns EGFR gene expression. EGFR transcripts were present at a 2.2 to 5.5 fold higher level MDBK cells expressing E5, E6 or E7 compared to control cells, whereas *EGFR* was under-expressed in BPV2 positive urinary bladder tumors of cattle. However, no BPV2 transcriptional activity was detected in these bladder tumors (Cota et al., 2015). This study aimed to set up an *in vitro* model to better understand the effect of BPV2 in bovine cells. However none of the putative BPV2 oncogenes induced transformation of bovine epithelial cells. Future research using other bovine cell lines, including fibroblast, could provide a more appropriate model for studying the role of BPV2 in naturally occurring tumors, and, in particular, how they might cooperate with the bracken fern's toxic compounds that are in the genesis of BEH.

Chapter VII – General Discussion and Conclusions

The studies contained within this thesis focused on the oncological process associated with BEH, especially on the participation of BPV2 in the development of UBT in the affected cattle.

In the first study we performed a histopathological characterization of the lesions found in the urinary bladders of BEH-affected cattle from the Azores Archipelago. The study of those lesions supported the significant neoplastic part of BEH. Although the majority of lesions corresponded to urothelial carcinomas, other histopathological types of tumors were also found. Our results were very similar to the ones published on a previous report regarding urinary bladder lesions of cattle from São Miguel Island (Carvalho et al., 2006). The expression of CK7 and CK20 in urothelial neoplasms was found to be maintained even in some invasive and undifferentiated tumors. Although some differences in the numbers of neoplastic cells expressing CK7 and CK20 were found, it seems that total loss of expression of these cytoskeletal proteins is a rare event in bovine urothelial tumors. These markers are commonly used in immunohistochemical panels to determine the origin of carcinomas in man (Jasik, 2010). Regardless of the lack of follow up studies, BEH can have substantial scientific interest, contributing for a better knowledge of lower urinary tract neoplasms in other species.

In the following two studies we attempted to clarify the role of BPV2 in the BEH-associated carcinogenesis process using samples collected in the Azores Archipelago major slaughterhouses, a Portuguese BEH endemic region. In the first study we sought to determine the prevalence of BPV2, and to quantify the viral loads present in the UBT of cattle. For these purposes we developed a TaqMan™-based qPCR system targeting specifically the BPV2's L1 and E5 genes. Furthermore, the iliac lymph nodes and normal areas of the urinary bladder surrounding the tumors were also tested in order to assess the distribution of BPV2 in tissues physically associated. The assessment of the possibility of BPV2 infection being productive was also evaluated on those tumors using the same qPCR system.

In the second study we examined the expression of cell-cycle controlling genes *TP53*, *MDM2* and *CCND1*, and a major growth factor receptor gene *EGFR*, in BPV2 positive and negative UBT. Relative quantification of mRNA was performed using qPCR, and protein expression was confirmed by immunohistochemistry.

BPV2 DNA was detected in at least one of the types of tissues tested (normal urinary bladder, UBT, iliac lymph node) both in animals with UBT (71%) as well as in those without signs of bladder neoplasia (54%). These findings support the suggestion that the

high prevalence of BPV might be the result of the high density of cows in a restricted geographical area as São Miguel or Terceira Islands (Pinto, 2010). Of all of the Portuguese territory, the Azores and Madeira Archipelagos have the highest concentrations of cow holdings (farms, cattle markets), and the Azores account for 11% of all Portuguese cow holdings (Baptista and Nunes, 2007). Regarding the urinary bladder tumors, no association was found between BPV2 DNA, or viral loads, and the histological type of lesion. In other studies similar results were obtained, leading to the suggestion that the histological features of the neoplasms arising from the urinary bladder of BEH-affected cattle might be related to factors other than BPV or bracken exposure (Borzacchiello, 2003; Borzacchiello et al., 2007) Thus, we suggest that BPV2 could not be unquestionably associated to the development of any specific type of tumor.

Although it has been suggested in a recent report that BPV2 urinary bladder infection can be productive, resulting in the assembly of virions in the urothelial neoplastic cells (Roperto et al., 2013), we did not detect L1 or E5 viral gene transcripts in the BPV2 positive lesions studied. Our findings were not surprising when taken in to account the features of the urothelium. The morphological and/or functional differences between the urothelium and the stratified squamous epithelium might be enough to disturb the necessary steps for a papillomavirus productive infection. Thus, our results suggested that the viral infection was an abortive one, BPV2 can indeed infect the urinary bladder but no viral particles are produced, as was previously stated (Campo et al., 1992; Yuan et al., 2007). Furthermore, a BPV2 abortive infection should more likely result in the development of urinary bladder neoplasms rather than a productive infection, since virus-induced cancers are supposed to occur at sites where a productive infections cannot be supported (Doorbar, 2006). The life cycle of HPVs is thought to be strictly dependent of the cell differentiation program of the host epithelia, and so is the viral gene expression program (Groves and Coleman, 2015). Briefly, it is proposed that HPVs infect the stem cells of the stratified epithelia which can become exposed as a result of microwounds. The number of viral genomes copies is maintained in the basal undifferentiated cells, being replicated as these cells divide. The expression of viral early genes takes place in the intermediate layer of the stratified epithelia. Amplification of the viral genome, expression of late viral genes and virion assembly only occur in more differentiated cells of the upper layers of the stratified epithelia (reviewed in Hong and Laimins, 2013). A quite similar life cycle is assumed to occur for most of BPV infections (Munday, 2014). The results obtained in the present work suggest that the urothelium, most probably due

to its morphological characteristics, might not support a productive BPV2 infection. Regarding the endothelial cells, which after neoplastic transformation give rise to the vascular tumors commonly identified in the urinary bladders of BEH-affected cattle, we propose that the infection that occurs is equally abortive.

The study of the expression of cell-cycle controlling genes and *EGFR* revealed a general overexpression of the *TP53*, *MDM2* and *CCND1* genes regardless of the tumor BPV2 status. These results suggest that the regulatory pathways in which these genes participate are affected in urinary bladder tumors of cattle. The cause of the gene overexpression can only be hypothesized since the status of these genes was unknown. The major finding within the scope of this study is the association between BPV2 positive tumors and lower *EGFR* gene expression levels. This is the first report regarding the possible effect of BPV on the expression the EGFR. The HPV associated transformation process is proposed to be linked with, to some extent, the physical association of E5 and EGFR (DiMaio and Petti, 2013). The E5 oncoprotein encoded by HPV16 was reported to induce EGFR overexpression in cervical cancer in women (reviewed in Soonthornthum et al., 2011). This is the opposite of what we found on bovine UBT. Furthermore, we did not detect viral gene transcription in any of the BPV2 positive tumors as mentioned above, suggesting that the possible action of BPV2 in the modulation of *EGFR* gene expression might not be linked to continuous viral oncoprotein expression. If this was the case, BPV2 neoplastic transformation would happen according to the "hit and run" carcinogenesis model. This model was proposed for the case of BPV4-associated neoplastic transformation, since in *in vitro* conditions, after the malignant phenotype was established, the viral genome was no longer detected in the transformed cells (Smith and Campo, 1988). Reports on the expression of BPV2's E5 and E7 oncoproteins in UBT of cattle and water buffalos were presented in different studies by the same group of authors, using PCR and immunohistochemistry techniques (Balcos et al., 2008; Borzacchiello, 2003; Borzacchiello et al., 2007; Maiolino et al., 2013; Roperto et al., 2013), which are in disagreement with the possible "hit and run" carcinogenic effect of BPV2.

It should be noted that, although not presented, the assessment of *PDGFR-β* gene expression by qPCR was attempted under the same conditions as of the other genes studied. The expression and role of PDGFR-β in BPV2-associated UBT has already been studied (Borzacchiello et al., 2003; Borzacchiello et al., 2006). The lack of soundness in the results obtained during the present study did not allow any strong conclusions to be produced on the expression of this gene. Nevertheless, in a BPV2 latency scenario the

implications of PDGFR-β in UBT development should also be pursued, and immunohistochemical detection of this growth factor receptor can be the best path for future studies.

When studying the BEH associated carcinogenesis it should bared in mind the strong and proven neoplastic potential of bracken fern and of its major toxin, ptaquiloside. It has been proposed that both bracken and BPV act synergistically in the development of UBT (Campo et al., 1992). In this proposed interaction the bracken's carcinogens seem to have a greater impact than BPV2, since UBT in cattle are very uncommon in geographic areas where bracken fern does not grow endemically (Meuten et al., 2004). Furthermore, the implementation of prophylactic measures eliminating bracken from the pastures has clearly contributed to reduce the number of UBT of cattle slaughtered at the São Miguel Island abattoir (Pinto et al., 2012; Pinto, 2010). Though we have reported a high prevalence of BPV2 in cattle reared in the Azores Archipelago, little is known on the prevalence of BPV2 in BEH-free regions. Without information on BPV prevalence in other regions where BEH is not a problem, it is challenging to assess the extent of the association between this viral agent and BEH. The presence of BPV2 DNA in normal urinary bladders, and the absence of BPV2 DNA in UBT, have led to the hypothesis that the viral participation is not required for the development of all UBT (Pinto, 2010). Additionally it is also possible that more permissive conditions created the ingestion of bracken allows BPV2 to infect the urinary of cattle without having any role on the neoplastic process that is undergoing (Munday, 2014).

On the third study we assessed the effects of BPV2's E5, E6 and E7 oncoproteins in *in vitro* conditions. The MDBK cell line was used in an attempt to replicate the *in vivo* conditions of BPV2-induced neoplastic transformation of urothelial cells. The construction of replicative defective recombinant retroviruses allowed a stable expression of BPV2's oncogenes. Despite the transcription of the putative BPV2's oncogenes, confirmed by qPCR analysis, no morphological changes were observed in the infected cells. Most of the *in vitro* studies on BPV-associated transformations were performed using BPV1. In the course of these studies the activities of BPV's oncoproteins were progressively uncovered. The expression of BPV1's E5 or E6 was proven to induce a cellular transformation phenotype in fibroblast cell lines of other species than bovine (Bergman et al., 1988; Petti and Ray, 2000; Tong and Howley, 1997; Yuan et al., 2011). On the other hand, BPV1's E7 oncoprotein seems to enhance the transformation

capabilities of E5 and E6 rather than having a strong transforming potential of its own (Bohl et al., 2001; DeMasi et al., 2007). The lack of the canonical pRB-binding domain in the E7 protein of BPV1 and BPV2 might have an influence on the oncogenic effects of this oncoprotein (Narechania et al., 2004). As BPV1 and BPV2 have identical E5 gene sequences, and the two E6 genes share an 84% amino acid identity, we estimated that our experiments with BPV2's E5 and E6 could also result in a cellular transformed phenotype. However, it seems that MDBK cells do not fully meet the requirements for BPV2 oncoprotein transformation, as was assumed after the confirmation of the lack of expression of PDGFR-β in these cells. Nevertheless, in our preliminary study we have found that the expression of BPV2's oncoproteins can influence cellular division rates and gene expression profiles. The most striking finding was the slower cell division rates induced by E7, paired with the highest expression levels of *TP53* tumor suppressor gene. This effect seems to be opposite to the expected actions of an oncoprotein. The exact molecular mechanisms by which BPV2's E7 induces *TP53* overexpression should be pursued.

Overall, the studies presented within this thesis suggest that the infection of urinary bladder of cattle by BPV2 is not sufficient for full neoplastic transformation. This infection seems to be abortive, and the hypothetical expression of viral oncoproteins should only occur in the initial steps of malignant transformation. The extent of the possible modulation of the carcinogenic process by BPV2 appears to be limited and it is still far from being fully understood. The effects of BPV2's oncoproteins should be further studied as other yet unknown functions might influence the neoplastic transformation pathways engaged by this infectious agent.

The studies developed within the scope of this thesis have raised new research questions in different scientific areas. The first area is genomics. A genomics approach could help to better understand the gene alterations that occur and lead to the development of UBT in BEH-affected cattle. The information gathered from gene alterations could be relevant in the comparative pathology framework.

The second area is BPV mediated cell transformation. The results obtained point that other oncoprotein functions might still be concealed. Bovine fibroblast cell lines should be more suitable than epithelial cells for BPV2 transformation assays.

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