ESCOLA UNIVERSITÁRIA VASCO DA GAMA

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HEAT SHOCK PROTEINS IN TRANSLATIONAL ONCOLOGY

Diana Filipa Moreira de Oliveira

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Diana Filipa Moreira de Oliveira
Master's degree student in Veterinary Medicine

Internal Supervisors

Professora Doutora Ana Calado

External Supervisors

Dr. Luís Carvalho
(Hospital Veterinário da Marinha Grande)

Dra. Barbara Karolczak
(Bath Vet Group)

Dr. Jeff Young
(Planned Pethood International)

Constituição do Júri

Presidente do Júri: Prof. Doutor Sérgio Sousa
Arguente: Prof. Doutora Ana Isabel Portela
Orientador: Prof. Doutora Ana Calado Lopes

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List of Abbreviations

ABD – ATPase Binding Domain

AIF – Apoptosis Inducing Factor

APC – Antigen Presenting Cell

CTVT – Canine Transmissible Venereal Tumor

HSPs – Heat Shock Proteins

HSR – Heat Shock Response

ICE – Intracutaneous Cornifying Epitheliomas

kDa – Kilodaltons

NK cells – Natural Killer cells

PBD - Peptide Binding Domain

SCC – Squamous Cell Carcinoma

sHSP – Small Heat Shock Proteins

TNF – Tumor Necrosis Factor

TPR – Tetratricopeptide Repeat

Heat Shock Proteins in Translational Oncology

Diana Oliveira [a], Ana Calado [a b]

[a] Departamento de Medicina Veterinária, Escola Universitária Vasco da Gama, Av. José R. Sousa Fernandes 197, Campus Universitário- Bloco B, Lordemão, 3020-210, Coimbra, Portugal (diana.m.oliveira@live.com.pt; anacaladolopes@gmail.com)

[b] Centro Veterinário Vasco da Gama, Av. José R. Sousa Fernandes 197, Campus Universitário- Bloco B, Lordemão, 3020-210, Coimbra, Portugal (anacaladolopes@gmail.com)

Abstract

Heat Shock Proteins are a group of proteins that is induced in response to stress and harmful situations. The several proteins that belong to this group are further divided into families, based on their molecular weight, distinguished by the terms HSP27, HSP40, HSP60, HSP70, HSP90 and an additional family of large HSPs.

This is a group of proteins that is constitutively expressed in normal cells, being responsible for the maintenance of the celular homeostasis. It is indeed their basal level of expression that sustains the pathways that occur in healthy intracellular environments. Since HSPs interact with multiple other proteins, they are referred to as “molecular chaperones”, while the proteins that they help receive the name of “client proteins”.

However, there are some HSPs that assist other HSPs. In this situation they are called “co-chaperones”, as in the case of HSP40 with both HSP70 and HSP90.

Although HSPs were first discovered after a heat shock, nowadays it is known that they can be induced by a variety of other stimuli. The work they do in stressful situations is to help the cells to restore their physiological environments, being fundamental for viability and survival of organisms.

Cancer cells also take advantage of these mechanisms. An oncologic process is a harmful situation, which culminates with the activation of HSPs, allowing cancer cells to survive to otherwise lethal conditions. They also contribute to the worst prognosis of the neoplastic disease.

A therapeutic approach that was invented by studying the role of HSPs in tumorigenesis was the creation of molecules that inhibit HSPs and their functions. This way, Translational Oncology has become a very important field, allowing nanoproteins, like HSPs, to be used as potencial anticancer targets. HSP90 is considered the perfect drug target among all HSPs, with its inhibitors being the ones with the most promising results. Because these drugs are still in the experimental phase, laboratory rodent and canine species have been used in several clinical trials of HSPs inhibitors.

Generally, HSPs are overexpressed in the majority of cancers, in both humans and animals. Given the epidemiological, morphologic and biological similarities that human beings share with companian animals, it was concluded that spontaneous tumors in this species provide the best model to study the disease in humans.

Key-words: “*Heat Shock Proteins*”, “*HSP27”,* “*HSP70*”, “*HSP90*”, “*HSP inhibitors*”, “*immunotherapy*”, “*cancer*” and “*animal models*”.

Resumo

Proteínas de Choque Térmico são um grupo de proteínas que é induzido em resposta ao *stress* e situações lesivas. As várias proteínas que pertencem a este grupo estão divididas em famílias, de acordo com o seu peso molecular. Podem ser distinguidas a HSP72, HSP40, HSP60, HSP70, HSP90 e uma família adicional de grandes HSPs.

Este é um grupo de proteínas que é constitutivamente expresso em células normais sendo responsável pela homeostase celular. É de facto o seu nível de expressão basal que sustenta os processos que ocorrem num ambiente intracelular saudável. Uma vez que as HSPs interagem com muitas outras proteínas, elas são referidas como "ajudantes moleculares", enquanto as proteínas que elas ajudam recebem o nome de "proteínas cliente".

Contudo, também existem HSPs que ajudam outras HSPs. Nesta situação elas são chamadas de "co-ajudantes", como no caso da HSP40 com a HSP70 ou HSP90.

Apesar das HSPs terem sido primeiramente descobertas após um choque térmico, atualmente sabe-se que podem ser induzidas por diversos outros estímulos. O seu desempenho em situações de *stress* é ajudar as células a restaurar o seu ambiente fisiológico, sendo fundamentais para a viabilidade e sobrevivência dos organismos.

Células cancerígenas também tiram vantagem destes mecanismos. Um processo oncológico é uma situação lesiva que culmina na activação das HSPs, que contribuem para os piores prognósticos nas doenças neoplásicas.

Uma abordagem terapêutica que surgiu com o estudo das HSPs no desenvolvimento tumoral foi a criação de moléculas que inibem as HSPs e as suas funções. Desta forma, a Oncologia Translacional tornou-se um campo muito importante, permitindo que nanoproteínas, como as HSPs, sejam usadas como potenciais alvos anti-cancerígenos. HSP90 é considerada o alvo terapêutico perfeito, entre todas as HSPs, visto que os seus inibidores demonstram os melhores resultados. Uma vez que estes fármacos estão ainda numa fase de experimentação, espécies de roedores e canídeos de laboratório têm sido usados em diversos ensaios clínicos de inibidores de HSPs.

Geralmente as HSPs encontram-se excessivamente expressas na maioria dos tumores, tanto em humanos como em animais. Considerando as semelhanças epidemiológicas, morfológicas e biológicas que os seres humanos partilham com os animais de companhia, espera-se que os tumores espontâneos destas espécies sejam o melhor modelo animal para estudar a doença em pacientes humanos.

Palavras-chave: "Proteínas de Choque Térmico", HSP27", "HSP70", "HSP90", "Inibidores de HSPs", "imunoterapia", "cancro" e "modelos animais".

Introduction

Organisms must survive to a variety of stressful conditions, so in response to stress and harmful situations, cells express Heat Shock Proteins (HSPs) (Nahleh, Tfayli, Najm, El Sayed, & Nahle, 2012; Richter, Haslbeck, & Buchner, 2010). HSPs were given this name because their expression was first discovered in patients that suffered from heat shock (Romanucci, Basto, & Della Salda, 2008; Soo, Yip, Lwin, Kumar, & Bay, 2008). The induction of HSPs in response to stress is named Heat Shock Response (HSR) (Richter et al., 2010).

HSPs are a group of highly conserved proteins, being expressed by eukaryotic and procariotic organisms (Kano, Abe, & Hasegawa, 2004; Maleki, Khosravi, Nasser, Taghinejad, & Azizian, 2016). They represent 5 to 10% of the total protein of a healthy cell, being one of the most abundant group of proteins found in living organisms (Chu et al., 2001; Soo et al., 2008).

Mammalian HSPs can be divided into six families, according to their molecular weight in kilodaltons (kDa): HSP40, HSP60, HSP70, HSP90, HSP100 (Ajayi et al., 2018; Chatterjee & Burns, 2017; Lianos et al., 2015; Romanucci et al., 2008; Scieglinska, Krawczyk, Sojka, & Gogler-Pigłowska, 2019; Soo et al., 2008). There is also another group of small HSPs (sHSP), whose molecular weight varies between 12 and 43 kDa, that includes HSP27 (Yun, Kim, Lim, & Lee, 2019). Each family is composed of several different proteins that share great resemblance in primary structure, enabling them to perform similar functions in the different subcellular compartments (Selvarajah et al., 2013).

HSPs with the highest molecular weight are ATP-dependent, while the smallest act in an ATP-independent way (Chatterjee & Burns, 2017). Their functions include helping in the formation of new proteins and being involved in the folding and refolding of the so called “client proteins”, acting as molecular chaperones (Romanucci, Marinelli, Sarli, & Della Salda, 2006;Shan et al., 2019). They are present in essential processes, such as: proliferation, transport, translocation, cell cycle control, angiogenesis or apoptosis (Nahleh et al., 2012). If after multiple insults, a protein becomes irreversibly damaged, HSPs help on its elimination, guiding it to the proteasomes (Penke et al., 2018; Scieglinska et al., 2019). The work HSPs do is based on maintaining celular homeostasis, which makes them an important defence mechanism, essential for normal cell viability, growth and survival (Nahleh et al., 2012; Yun et al., 2019).

Not all HSPs are stress inducible (Whitesell & Lindquist, 2005). In some physiological situations, like aging, HSPs can be constitutively expressed (Murshid, Eguchi, & Calderwood, 2013). This means that cells are able to synthesize HSPs due to both normal and stressful reasons (Lang et al., 2019).

Currently, a big variety of environmental insults are known to induce HSP response, such as: hypherthermia, oxidative stress, radiation (Nahleh et al., 2012), fever, hypoxia, surgery and emotional stress (X. Wang, Chen, Zhou, & Zhang, 2014).

When a cell is under an aggression or insult, the activation of the HSPs pathways is what allows cells to survive otherwise lethal conditions (Lang et al., 2019). This is the reason why HSPs expression is

abnormally higher in cancer cells (Lang et al., 2019). Cancer cells undergo extensive intracellular changes, which mean they are constantly subjected to a variety of different stressors (Nahleh et al., 2012). Their higher metabolic requirements make them dependent on HSPs, because of the several client proteins that will help cancer cells to survive (Chatterjee et al., 2017). This way, the cytoprotective role of HSPs becomes essential for tumor progression and differentiation (Ciocca & Calderwood, 2005). HSPs not only allow cancer cells to survive, but also give them unlimited replication and metastatic potential, which ultimatly leads to resistance to chemotherapy (Lianos et al., 2015).

Abnormal HSPs levels have been observed in a wide variety of animal neoplasms, which led to the idea that they could be used as biomarkers for the diagnosis and prognosis of the disease (Romanucci et al., 2008; Romanucci et al., 2012). At the same time they emerge as new molecular targets for anti-cancer therapy, based on their inhibition (Chatterjee & Burns, 2017; X. Wang et al., 2014).

Similar expression patterns are detected between human and animal neoplasms (Romanucci et al., 2006). These parallel findings highlight the relevance of studying the multiple roles that HSPs have in carcinogenesis, using animal models (Romanucci et al., 2012). This way, animal models function as an additional source of information for clinical cancer research (Talmadge, Singh, Fidler, & Raz, 2007; Tarone et al., 2019; Vail & Macewen, 2000).

The aim of this review is to explain what HSPs are and by which mechanisms they interact with several cellular pathways. While exploring the theme it was decided to treat each family individually, starting with the HSPs with the lowest molecular weight. Then it will follow the HSP40, HSP60, HSP70 and, lastly, the HSP90. In each family it will be demonstrated how HSPs could function as biomarkers in the diagnosis and prognosis of neoplasms, as well as to act as molecular targets in future therapeutics. This review culminates with an explanation about the importance of murine models and spontaneous canine models and how thay can increase our knowledge about oncologic disease, in both Human and Veterinary Medicine.

HSP27

HSP27 is a member of the small heat shock proteins (sHSP), being the most studied member of this family (Chatterjee & Burns, 2017). It is mostly found in the cytosol of organs associated with estrogen receptors (Castagnetta & Carruba, 1995; Mlynarczyk-liszka et al., 2009; Nahleh et al., 2012).

As shown in Figure 1, the HSP27 structure consists of a conserved α-crystallin domain and a variable C- and N-terminal region (Singh, Sharma, & Tiwari, 2017). The C- and N- terminals, although different in sequence and length, are essential for the correct folding of client proteins (Ajayi et al., 2018). In HSP27, like in other sHSP, the C-terminal tail acquires a flexible conformation (Arrigo, 2018).

Figure 1 - HSP27 structure. In blue the N- and the C- variable terminal domains. In orange the α-Crystallin domain. Adapted from Wu et al., 2017.

In canines, the HSP27 is formed by 209 amino acids and shares 86 to 89% similiarities with the human, hamster, mouse and rat small HSP (Romanucci et al., 2005).

In bovines, after sequencing their genome, it was discovered that this species has 10 genes for small HSPs, scattered across nine chromosomes (Ajayi et al., 2018). Similar results have been reported in humans, suggesting that the genes of both species have been preserved since the time of their common ancestor (Ajayi et al., 2018). Like in other species, the bovine small HSPs structure is also composed by a conserved alpha crystallin domain and variables N- and C-terminal regions (Ajayi et al., 2018).

HSP27 is directly linked to some types of cell differentiation, like keratinocytes (Kiriyama, Oka, Takehana, & Kobayashi, 2001). Because it is constitutively expressed in normal epidermis, HSP27 is seen as a chaperone for cornification (Romanucci et al., 2005; Scieglinska et al., 2019).

In the same way, HSP27 is also responsible for maintaining the cytoskeleton (Graceffa, 2011). It is highly associated with several cytoskeletal proteins, like actin, which requires its protection during stress (Concannon, Gorman, & Samali, 2003; Romanucci et al., 2006).

HSP27 displays strong anti-apoptotic properties (Heinrich, Tuukkanen, Schroeder, Fahrig, & Fahrig, 2011). It is able to stop apoptosis at different stages, since it interacts with several client proteins implicated in the apoptotic process (Concannon, et al, 2003).

In normal cells, HSP27 expression is low or undetectable (Nahleh et al., 2012). However, high protein levels have been identified in some cancer types (Concannon, et al, 2003; Gibert et al., 2011). The

tumorigenic potential of HSP27 is related to tumor invasiveness, metastasis, drug resistance and ultimately lower survival rates (Calderwood, 2016; J. C. Heinrich et al., 2016).

In Veterinary Medicine, mammary tumors are the most common malignant neoplasm in the bitch (Romanucci et al., 2006). They share a lot of similarities, both pathological and clinical, with the human breast cancer (Kumaraguruparan, Karunagaran, Balachandran, Manohar, & Nagini, 2006).

HSP27 was identified in canine mammary gland tumor, but with low positive levels (Romanucci et al., 2006). When present, it was mostly found in infiltrating tumor cells that invaded the surrounding stroma (Romanucci et al., 2006). HSP27 expression is thus related to invasive tumor behavior, shorter survival rates and poor prognosis (Romanucci et al., 2006).

HSP27 is also expressed in multiple animal skin cancers, such as Intracutaneous Cornifying Epitheliomas (ICE) (Romanucci et al., 2005). In this benign neoplasm, canine HSP27 was associated with areas of proliferating epithelial cells (Romanucci et al., 2005).

Squamous Cell Carcinoma (SCC), a more aggressive type of skin tumor, was also investigated (Romanucci et al., 2005). Here, HSP27 was less intense, when compared to ICE or normal skin, however, it was still positive in keratinizing cells (Romanucci et al., 2005; Scieglinska et al., 2019). One important discovery was that HSP27 is accumulated in neoplastic cells, based on the level of keratinocyte differentiation (Romanucci et al., 2006). This means that HSP27 is highly expressed in well differentiated carcinomas, but its levels are low in poorly differentiated ones (Romanucci et al., 2005).

These findings are in accordance with those reported from murine and human skin, where one essay tried to understand how HSP27 changes in mouse skin cells, after chronic exposure to UV-B radiation (Kiriyama et al., 2001). In this study, as the SCC progressed, HSP27 expression got weaker, to a point where it was not even detected (Kiriyama et al., 2001; Scieglinska et al., 2019). This way, and similarly to what was mentioned for canine SCC, in mouse, the HSP27 expression also decreases in less differentiated tumors (Kiriyama et al., 2001).

The results of these studies prove that HSP27 expression is directly correlated with keratinocyte differentiation, but not with tumor progression (Kiriyama et al., 2001 Romanucci et al., 2006). It can be concluded that HSP27 acts as a marker for human, canine and mouse skin differentiation, with its absence being a sign of epidermal malignancy (Kiriyama et al., 2001; Romanucci et al., 2005).

Mouse models were used to study other types of cancer, such as colon carcinomas (Garrido et al., 1998). On the former, HSP27 levels were increased, being associated with malignant characteristics (Garrido et al., 1998). This proves that different tumors are able to modulate HSP27 expression in different ways (Kiriyama et al., 2001).

HSPs can be involved not only in tumors of soft tissue organs, but also in bone neoplasms (Romanucci et al., 2012; Selvarajah et al., 2013). However, HSP27 only showed weak levels of expression in canine appendicular osteosarcoma (Romanucci et al., 2012). On the other hand, HSP10 and HSP22, two other

members of the small HSPs family, are the ones that are overexpressed in the worst cases of canine osteosarcoma, being associated with a non-response to chemotherapy and shorter survival times (Selvarajah et al., 2013).

All of the small HSPs characteristics mentioned above are summarized in Table 1.

Table 1 - Main characteristics of small HSPs, with emphasis on their role in the neoplasms where they were found

Small HSPs					
Functions	**Situation**	**Tumor types**	**Species**	**Tumor Origin**	**Importance in Oncology**
-Protein folding; - Cell growth; - Cell migration; (Nahleh et al., 2012) - Keratinocyte differentiation; (Kiriyama et al., 2001). -Maintenance of the cytoskeleton (Graceffa, 2011)	Physiological				
- Inhibition of apoptosis (Concannon, et al, 2003)	Physiological / Pathological				
- Tumor invasion; - Metastasis; - Drug resistance (Calderwood, 2016; J. C. Heinrich et al., 2016)	Pathological	Mammary Gland Cacinoma	Canines (Romanucci et al., 2006)	Spontaneous (Necropsy + Surgical Samples)	Indicative of poor prognosis
		Intracutaneous Cornifying Epitheliomas	Canines (Romanucci et al., 2005)	Spontaneous (Biopsy + Surgical Samples)	Marker of keratinocyte differentiation Absence is a sign of poor prognosis
		Squamous Cell Carcinoma	Canines (Romanucci et al., 2005)	Spontaneous (Biopsy + Surgical Samples)	
			Murines (Kiriyama et al., 2001)	Induced (UV-B stimulation)	
		Osteosarcoma	Canines (Selvarajah et al., 2013).	Spontaneous (Surgical Samples)	Indicative of poor prognosis

One of the characteristics of HSP27 is being able to suffer oligomerization (interaction of more than one polypeptide to form a quaternary structure) (Nirwan & Kakkar, 2018) and phosphorylation (addition of a phosphate group) (Ardito, Giuliani, Perrone, Troiano, & Muzio, 2017). The oligomerization is fundamental in the regulation of HSP27 chaperone activity, being necessary for the binding of client proteins (Arrigo, 2018; Scieglinska et al., 2019). Oligomers can reach up to 1000kDa, with the dimer being the most important form of HSP27 oligomers (X. Wang et al., 2014).

It was discovered that, in cattle, small HSPs are also hydrophilic, which increases its functional ability to oligomerize and, consequently, to bind to client proteins (Ajayi et al., 2018)

HSP27 is associated with several isoforms, each one related to a different stage of phosphorylation (Romanucci et al., 2005). HSP27 also phosphorylates in response to stress and some of its phosphorylation patterns are even related to more aggressive types of tumors (Nahleh et al., 2012).

HSP27, like the others HSPs, has been proposed to be a potential target for chaperonotherapy, which means that the use of molecular chaperones in therapeutics (Rappa et al., 2012). HSP27 depletion can lead to tumor regression (X. Wang et al., 2014). However, HSP27 inhibitors still have some limitations, such as the fact that this chaperone (like others small HSPs) is ATP-independent, which makes it a challenge for drug targeting (Nahleh et al., 2012).

RP101 (also known as Bromovinyldeoxyuridine, Brivudine or BVDU) is an exemple of a HSP27 inhibitor (Chatterjee & Burns, 2017). This molecule binds to the shock protein, disrupting its interactions with several of its clients (Heinrich et al., 2011). By using rat models, it was discovered that RP101 is not effective on its own and should be combined with a classic cytotoxic drug, to have the potential of improving chemotherapy (Heinrich et al., 2011).

PA11 or PA50 are examples of peptide aptamers specific for HSP27 (Gibert et al., 2011). Peptide aptamers are defined as small amino acids sequences, commonly used to prevent interactions between proteins (Schmidt & Debant, 2013). PA11 acts by preventing the HSP27 oligomerization, while PA50, through a different mechanism, inhibits HSP27 dimerization (X. Wang et al., 2014). Both studies used mice bearing xenografts, where the peptide aptamers were effective in reducing tumor growth (Gibert et al., 2011).

HSP40

The HSP40 family constitutes the largest and most diverse group of HSPs, containing the greatest number of members (J. Wu et al., 2017). HSP40 members are divided into three subclasses, namely type I (DNAJA), type II (DNAJB) and type III (DNAJC) (Chatterjee & Burns, 2017; J. Wu et al., 2017). This classification is based on the presence or absence of domains, when compared to the *Escherichia*

coli gene *dnaJ* (Ajayi et al., 2018). This means that the *E. coli* bacteria is what is currently used to define the DNAJ family (Cheetham & Caplan, 1998).

Type I (DNAJA) consists of four members, all being very similar to the *E.coli* DNAJ (Kampinga et al., 2009). They are formed by a N-terminal J-domain, a glycine/phenylalanine (G/F) rich region, a cysteine repeat region (also known as Zinc finger) and a carboxil-terminal (Sterrenberg, Blatch, & Edkins, 2011). In Type II, DNAJB members have the same core structure as the ones in type I, except they lack the cysteine repeat region (Tamadaddi & Sahi, 2016). At last, the type III (DNAJC) has a completely different structure from the other two groups, as it is only formed by the J-domain, which can be located anywhere along the protein (Sterrenberg et al., 2011). The structure of the three types of HSP40 is schematized in Figure 2.

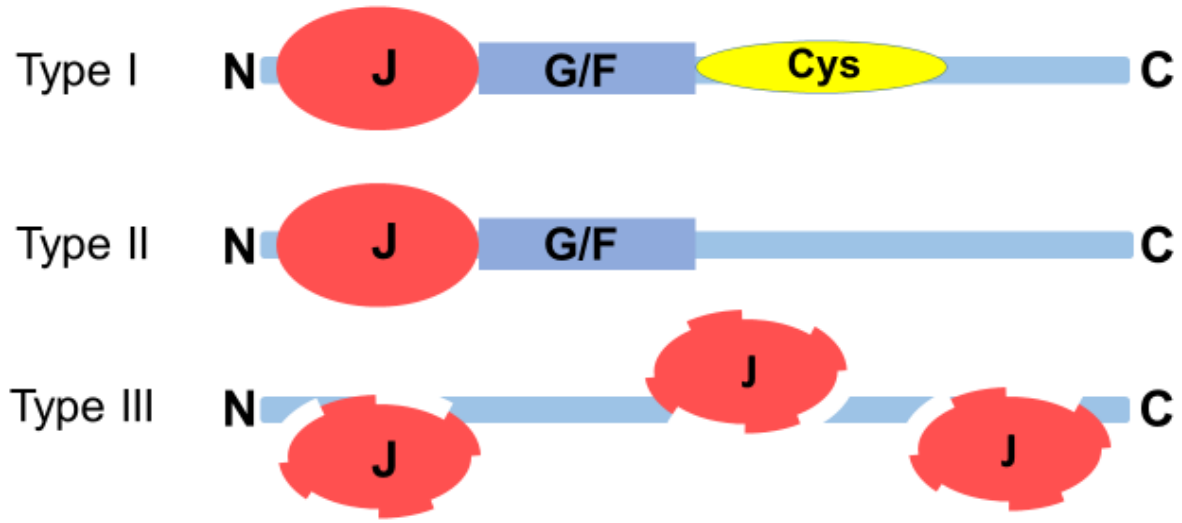

Figure 2 - Structure of the diverse types of HSP40. In red the J-domain, in blue the Glycine/Phenylalanine rich region and in yellow the Cysteine domain. Adapted from Wu et al., 2017.

The characteristics that define HSP40 are present in several mammal species (Ajayi et al., 2018). In bovines, for example, were found a total of 43 genes for the DNAJ familly, divided in the same three subclasses mentioned before (Ajayi et al., 2018). Type I includes four genes (DNAJA1, DNAJA2, DNAJA3 and DNAJA4), the type II has 12 genes, and lastly, in type III belong 27 genes (Ajayi et al., 2018). Generally, cattle HSP40 follows the same structural rules described in other species (Ajayi et al., 2018).

The J-domain, present in all HSP40, is a highly conserved region, occurring in all organisms (Sterrenberg et al., 2011). Its structure consists of four α hélices (Minami, Höhfeld, Ohtsuka§, & Hartl, 1996). Helix 2 and 3 run antiparallel, forming a groove in between. In the groove is located the histidine-proline-aspartatic (HPD) region (Qian, Patel, Hartl, & Mccoll, 1996). Lastly, helix 1 and 4 are strategically positioned in a way that conterbalance the other two (Sterrenberg et al., 2011).

One of the functions of HSP40 isoforms is to serve as co-chaperones, where they assist other HSPs, like HSP70 and HSP90, in their chaperone functions (J. Wu et al., 2017; Mitra, Shevde, & Samant, 2009).This might be the reason why HSP40 has such a large number of genes, because they are all necessary to stabilize the multiple interactions between HSP70 and their various client proteins (Ajayi et al., 2018).

The DNAJ J-domain, or more specifically its HPD region, is the most important element for the co-chaperone functions (Cheetham & Caplan, 1998; Tamadaddi & Sahi, 2016). This is the region that mediates HSP40 interactions with HSP70 (Sojourner et al., 2018). In the same way, the Cys repeat region, together with the C-terminal area, are part of the DNAJ substrate binding domain, being also necessary for peptide presentation to the chaperones (Cheetham & Caplan, 1998).

Although the information available about HSP40 is very limited, their isoforms are still regarded as important members of cancer development (Mitra et al., 2009; Yun et al., 2019). Targeting HSP40 should be a new approach in the development of anticancer treatments. (Sterrenberg et al., 2011). Even though some HSP40 act as tumor suppressors, the therapeutic approach may involve the induction of their expression, rather than inhibiting them (Sterrenberg et al., 2011).

HSP60

The HSP60, in eukaryotes, is typically found in the mitochondria, receiving the name of chaperonin. (Lianos et al., 2015). However, it can also be found in the cytosol and in the cell surface (J. Wu et al., 2017).This way, HSP60 is able to bind with both endogenous and exogenous proteins, while participating in intracellular and extracellular pathways (Cappello, Macario, Marasà, Zummo, & Macario, 2008).

Like other chaperones, HSP60 also has a role in polypeptide folding, since it helps newly formed proteins to reach their mature conformations (Meyer et al., 2003). Some of its functions are also related to carcinogenesis, specifically with tumor survival and proliferation (Wyzewski, Gregorczyk, Szczepanowska, & Szulc-Dabrowska, 2018).

HSP60 was identified in the cytoplasm of Canine Transmissible Venereal Tumor (CTVT) cells (Chu et al., 2001). Unexpectedly, this protein was especially increased in CTVT cells during regression phase, compared to the ones in progression phase (Chu et al., 2001). This can predict that HSPs are not only an indicative of bad prognosis, but can also be associated with tumor regression (Chu et al., 2001). Further studies are still necessary to prove this argument (Chu et al., 2001).

HSP60 was also found in canine osteosarcoma, being significantly increased in tumors that had low life expectancies, contributing to the aggressive phenotype (Selvarajah et al., 2013). HSP60 highlights the important role that HSPs could have in the prognosis of canine osteosarcoma, since it correlates with

shorter survival times and poor clinical outcomes (Selvarajah et al., 2013). Similar results have been reported in human osteosarcoma, with HSP60 seen as a potential diagnosis marker (Moon et al., 2010). These discoveries suggest that HSP60 should be considered as a new target for osteosarcoma anticancer therapy, with preclinical tests performed in dogs (Selvarajah et al., 2013). HSP60 functions in both normal and cancer situations are listed in Table 2.

Table 2 - Main characteristics of HSP60, taking into consideration its role in both normal and cancer situations

HSP60					
Functions	**Situation**	**Tumor types**	**Species**	**Tumor Origin**	**Importance in Oncology**
- Protein folding (Meyer et al., 2003).	Physiological				
- Maturation of dendritic cells; - Activation of T-cells (Feng et al., 2002) - Antigen presentation (Cappello et al., 2008).	Physiological / Pathological				
- Tumor proliferation; - Metastasis; - Tumor survival (Wyzewski et al., 2018)	Pathological	Transmissible Venereal Tumor	Canines (Chu et al., 2001).	Induced (CTVT cells inoculated in dogs)	Possible indicative of good prognosis
		Osteosarcoma	Canines (Selvarajah et al., 2013).	Spontaneous (Surgical Samples)	Indicative of poor prognosis; Target for anticancer therapy

HSP60 is not exclusively intracellular, since it can also be present in the cell surface of some mammal cell lines (Soltys & Gupta, 1997). In this location, HSP60 is known to interact with the immune system, causing the maturation of dendritic cells and the generation of an antitumor T-cell response (Feng, Zeng, Graner, & Katsanis, 2002).

Extracellular HSP60 also works with the immune system, specifically in the antigen presentation (Cappello et al., 2008). Lastly, HSP60 also circulates in the peripheral blood, but not much information is available regarding the impact that blood location has in cancer (Cappello et al., 2008).

HSP60 has the potential to be used as a target in anticancer therapy (J. Wu et al., 2017). One of the proposed strategies is to be used as an adjuvant in vaccines, because vaccines with HSP60 have a

more potent immunotherapeutic effect, compared with the administration of the antigen without the HSP (Huang et al., 2007).

HSP70

HSP70 is a broad designation that includes a large group of molecular chaperones, all weighing 70KDa (Murphy, 2013). The following proteins are the most important examples: HSP72 (also called HSPA1), HSP73 (also called HSC70 or HSPA8), Grp78 (also called Bip) and mtHSP70 (also called Mortalin) (J. Wu et al., 2017; Yun et al., 2019).

HSP72 is the stress-inducible protein, which means that its expression is mainly observed under stressful situations (Multhoff et al., 1995). On the other hand, HSP73 is ubiquitously expressed in multiple organs and tissues, being present in both normal and stress situations (X. Wang et al., 2014). For example, in healthy dogs, HSP70 was detected in mononuclear cells of peripheral blood, stomach samples (Kano et al., 2004) or in the upper strata of epidermis (Romanucci et al., 2005).

The HSP70 structure is repeated among humans, canines, bovines and mice, because they all share a 90 to 95% similarities for the HSP70 genes (Kano et al., 2004). This suggests that HSP70 is able to play analogous functions in multiple animals (Kano et al., 2004). In Veterinary Medicine it was discovered that the canine HSP70 is composed by 640 amino acids and has four different regions: a N-terminal ATPase binding domain (ABD), a peptide binding domain (PBD), a C-terminal domain and, lastly, the "EEVD" highly conserved terminal sequence (Kano et al., 2004).

On the one hand, the ABD domain includes the ATPase pocket, where proteins usually bind to regulate the ATPase activity (Tamadaddi & Sahi, 2016). On the other hand, the PBD domain is used for substrate binding, being responsible for several of the HSP70 chaperone functions, like protein folding (Goloudina, Demidov, & Garrido, 2012). Additionally, the TKD, which is an extracellular epitope bound to the HSP70 membrane, can also be found in the PBD (Gastpar et al., 2004). The HSP70 structure is represented in Figure 3.

In bovines, 10 genes that encode HSP70 proteins were found (Ajayi et al., 2018). All cattle HSP70 family members appear to have similar functions, supporting the theory that HSP70 functions are transversal across species (Ajayi et al., 2018). In the same way, these stress proteins were also associated with thermal stability, confirming once more the role of HSPs in the Heat Shock Response (Ajayi et al., 2018).

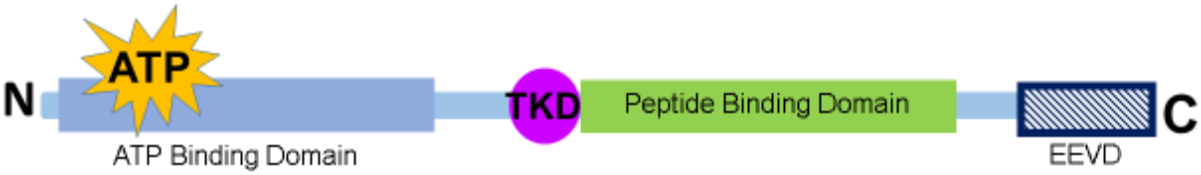

Figure 3 - HSP70 structure. In blue the ATP Binding Domain with the ATP pocket, in purple the TKD peptide, bound to the Peptide Binding Domain in green. Lastly, in dark blue the EEVD terminal sequence. Adapted from Chatterjee & Burns, 2017.

Most HSPs do not work alone (Sterrenberg et al., 2011). For example, the chaperone function of HSP70 is mediated by interactions with its co-chaperones, which increase or decrease the HSP70 ability to bind to the substrate (Radons, 2016). Co-chaperones are, thus, key regulators of the chaperone activity (Sterrenberg et al., 2011).

HSP70 co-chaperones are divided into three groups (Goloudina et al., 2012). The first group are the J-domain co-chaperones, like HSP40 (X. Wang et al., 2014). As mentioned previously, the J-domain of HSP40 is essential for the binding to HSP70 (Qiu, Shao, Miao, & Wang, 2006; Sojourner et al., 2018). As a co-chaperone, HSP40 regulates a big variety of biological functions (Tamadaddi & Sahi, 2016). However, one of its biggest roles is to assist in the folding of new or denatured polypeptides (Jiang, Rossi, & Kalodimos, 2019). For this to happen, HSP40 needs to bind to a specific client protein, presenting it to the substrate binding domain of HSP70 (Qiu et al., 2006). HSP70, which works in an ATP-dependent way, connects with the HPD region of HSP40 J-domain, whenever it is in the HSP70-ATP complex state (Sterrenberg et al., 2011).

The second type of HSP70 co-chaperones are the Nucleotide Exchange Factors (X. Wang et al., 2014). They help to achieve the ATP conformation, during HSP70 client protein folding (Goloudina et al., 2012; Radons, 2016; Richter et al., 2010). The role of HSP70 co-chaperones in protein folding is explained in Figure 4.

The last HSP70 co-chaperones are the Tetratricopeptide Repeat (TPR) Domains, such as Hop (a HSP70-HSP90 organizing protein) (X. Wang et al., 2014). This molecule binds to the C-terminal EEVD domain, presented in HSP70 and HSP90 (Brinker et al., 2002).This way, HOP is a co-chaperone that has the unique ability of interacting with both chaperones (Lang et al., 2019; Schopf, Biebl, & Buchner, 2017; Sterrenberg et al., 2011).

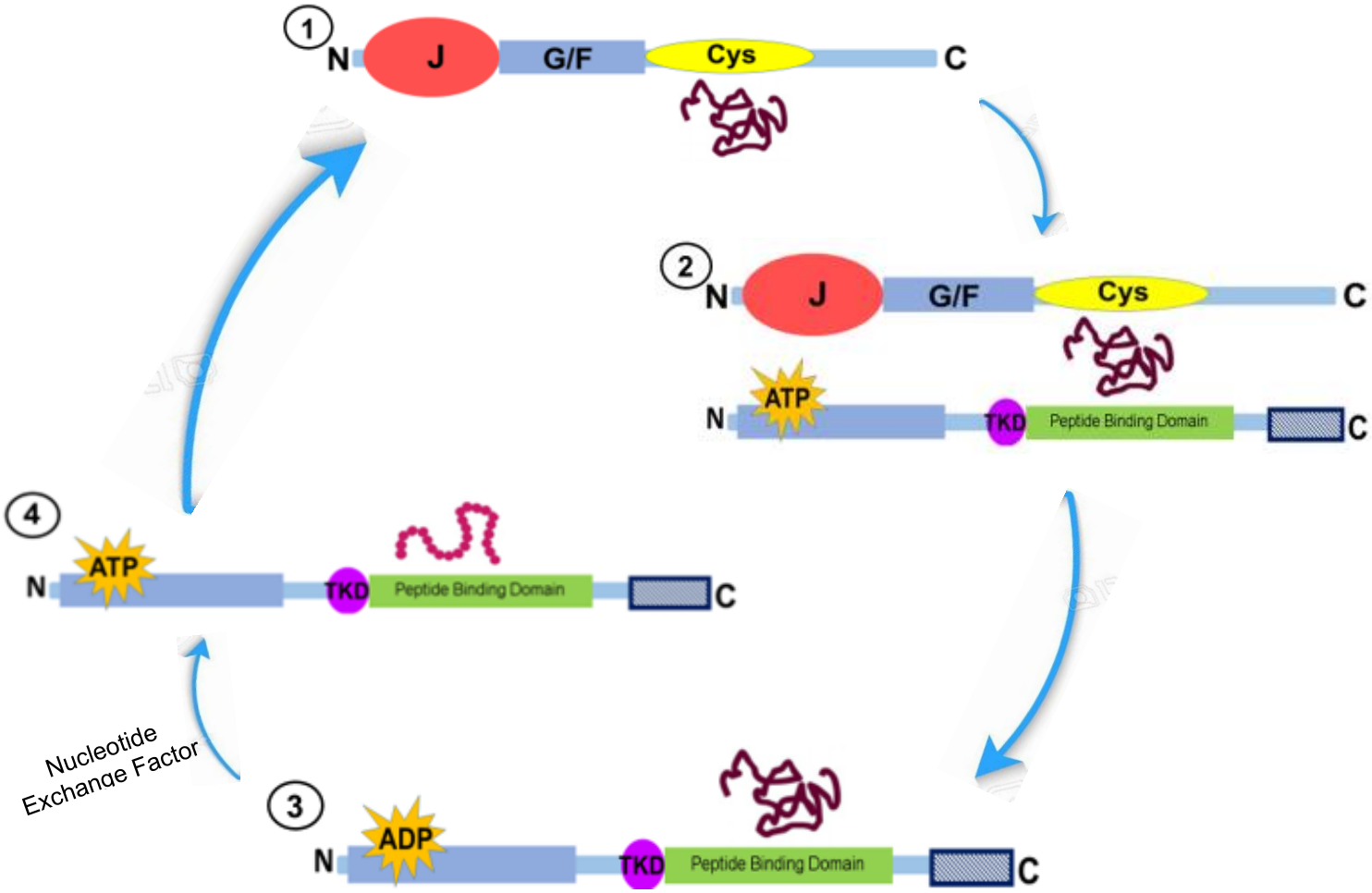

Figure 4 - Schematic representation of HSP70 protein folding, assisted by its co-chaperone HSP40 1 - HSP40 binds to a misfolded protein. 2 - Then HSP40 delivers the misfolded protein to HSP70, stimulating the hydrolysis of ATP to ADP in the ATP pocket of HSP70. 3 - HSP40 leaves the complex while the misfolded protein stays tightly binding to the ADP form of HSP70. 4 - With the help of a Nucleotide Exchange Factor, the ATP in HSP70 is regenerated and the client protein is finally released with a normal conformation. Adapted from Sterrenberg et al., 2011.

Another characteristic of HSP70 is that, not only does this protein have several co-chaperones, but it itself acts as a co-chaperone for another HSPs, namely HSP90 (Dahiya et al., 2019). In the presence of Hop, HSP70 together with HSP40, transfer unfolded proteins to HSP90, ensuring that client proteins reach their final maturation (Dahiya et al., 2019; Schopf et al., 2017).

Similarly to what happens with other HSPs, HSP70 levels are also high in cancer cells and are related with tumorigenic potential (Lianos et al., 2015; Murphy, 2013; Radons, 2016). Although the exact role of HSP70 in cancer is still unknown, it is thought that it offers a survival advantage to cancer cells, through the suppression of cells endogenous protection mechanisms, like apoptosis or senescence (Nahleh et al., 2012).

Several species of mammals, like humans or rats, show an increase in the nuclear translocation of HSP73 and HSP72, during the S-phase of cell cycle (Romanucci et al., 2008). HSP73 can be even

found during metaphase (Romanucci et al., 2006). This suggests that HSPs are also able to control cell cycle, by interfering with mitogenic pathways (Romanucci et al., 2008).

High levels of HSP70 were identified in Canine Transmissible Venereal Tumor (CTVT) (Chu et al., 2001). This characteristic can also confirm the strong role that HSPs play in cell protection, since CTCT cells are very resistant to high temperatures: it is the overexpression of shock proteins that makes them heat resistant (Chu et al., 2001). No HSP was detected in any of the other canine tumors studied by this essay, such as: nasal SCC, ovarian fibroadenoma or sertoli cell tumor (Chu et al., 2001). Based on this information, it was even suggested that HSP70 could be a potential biomarker to identify CTVT patients (Chu et al., 2001).

However, high levels of HSP70 were later discovered in canine mammary tumor cells (Romanucci et al., 2006). HSP72, the stress inducible one, showed a higher expression in mammary carcinomas, being associated with invasive characteristics of this type of tumor (Romanucci et al., 2006). On the other hand, HSP73, the constitutively expressed HSP70, had the highest levels in the normal mammary gland (Romanucci et al., 2006). However, unlike most HSPs, its expression decreased in neoplastic tissues (Romanucci et al., 2006). Although HSP73 does not seem to be related with invasion, it was still detected in inflammatory areas and metastatic cells, being necessary for the occurrence of lymphatic emboli in canine mammary carcinomas (Romanucci et al., 2006).

HSP70, together with some apoptotic proteins, were found to be altered, both in human and canine mammary tumors (Kumaraguruparan et al., 2006). The way that these proteins changed is very similar in both species, suggesting that they share the same mechanisms to evade apoptosis in this type of neoplasms (Kumaraguruparan et al., 2006).

HSP72 and HSP73 were also studied in canine skin neoplasms (Romanucci et al., 2005). Similarly to what was mentioned for HSP27 in Squamous Cell Carcinoma, HSP72 also had different expression patterns for well differentiated and moderately differentiated SCC (Romanucci et al., 2005). Its reactivity was more intense in well-differentiated carcinomas and reduced in moderately differentiated tumors (Romanucci et al., 2005). HSP73, on the other hand, showed opposite expressions, since it was less intense in well-differentiated SCC and slightly increased in moderately differentiated SCC (Romanucci et al., 2005). Nevertheless, unlike HSP27, the involvement of HSP70 family members in tumor cell differentiation is still very controversial (Scieglinska et al., 2019).

In bone tumors, HSP72 expression was significantly associated with higher tumor grades, in both human and dog osteosarcoma, suggesting an association with poor prognosis (Romanucci et al., 2012). Interestingly, HSP73 also showed high positive levels in canine osteosarcoma, as well as in metastatic emboli (Romanucci et al., 2012). This way, HSP73 with its constitutive properties, must confer proliferative advantages to bone tumor cells (Romanucci et al., 2012). Mortalin, the HSP70 with mitochondrial location, is another protein that has also been associated with low survival in canine osteosarcoma (Selvarajah et al., 2013). HSP70 main roles in tumors ar are described in Table 3

Table 3 - Main characteristics of HSP70 and its role in tumor development.

HSP70					
Functions	**Situation**	**Tumor types**	**Species**	**Tumor Origin**	**Importance in Oncology**
-Protein folding; - Client protein maturation (Jiang et al., 2019)	Physiological				
- Inhibition of apoptosis; - Inhibition of senescence (Nahleh et al., 2012)	Physiological / Pathological				
- Interfering with mitosis (Romanucci et al., 2008); - Antigen presentation - Activation of T-cells (Guzhova et al., 2016); - Tumor proliferation; - Metastasis; - Drug resistance (Radons, 2016)	Pathological	Mammary Gland Carcinoma	Canines (Kumaragurupar an et al., 2006; Romanucci et al., 2006).	Spontaneous (Necropsy + Surgical Samples)	Role in carcinogenes is and metastasis
		Squamous Cell Carcinoma	Canines (Romanucci et al., 2005).	Spontaneous (Biopsy + Surgical Samples)	---
		Transmissible Venereal Tumor	Canines (Chu et al., 2001).	Induced (CTVT cells inoculated in dogs)	Once hypothesized as a biomarker for the disease
		Osteosarcoma	Canines (Romanucci et al., 2012).	Spontaneous (Surgical Samples)	Indicative of poor prognosis

One important thing to take into consideration is that the presence of the same HSP in multiple histological cancer types, proves that they can´t be used as a marker to any specific neoplasm (Romanucci et al., 2008).

HSP70 inhibitors are divided into three different categories: small molecule inhibitors, protein aptamers and antibody treatments (X. Wang et al., 2014).

In the small molecule inhibitors can be included the ADD70, an Apoptotic Inducing Factor (AIF) derived peptide (X. Wang et al., 2014). Both ADD70 and AIF are two molecules that share an amino acid sequence, which allows them to attach to the peptide binding domain of HSP70 (Schmitt et al., 2003). ADD70 is, thus capable of binding to HSP70, neutralizing its chaperone functions and allowing cancer cells to undergo apoptosis (Radons, 2016). In an *in vivo* study using rats with colon carcinoma and mice with melanoma, it was concluded that ADD70 is capable of delaying tumor growth and reduce metastasis (Schmitt et al., 2006). In addition, ADD70 was also tested in combination with the chemotherapeutic, cisplatin. The two drugs led to a complete regression of neoplasms on murine models, but only when cisplatin was administered early in tumor development (Schmitt et al., 2006).

A17 is a HSP70 inhibitor that belongs to the protein aptamer category, being the most effective (Chatterjee & Burns, 2017). It binds to the ATPase domain, disrupting its functions (Radons, 2016). It also shows strong anti-cancer properties in mice models (Rérole et al., 2011). Its apoptotic potential is increased if used in combination with antineoplastic drugs, like cisplatin (Rérole et al., 2011).

cmHsp70.1 is a new monoclonal antibody that binds to TDK epitopes, whenever these are exposed on the surface of tumor cells (Stangl et al., 2011). This generates an antibody dependent cellular cytotoxicity (Radons, 2016). Only cancer cells express HSP70 with TKD in their membranes, making this epitope a tumor specific marker that overcomes the disadvantage of a lack of cancer biomarkers (X. Wang et al., 2014).

The TKD epitope is involved in other immunotherapeutic tools (Chatterjee & Burns, 2017). Whenever HSP70 appears on the outer membrane of cancer cells, exposing its TKD peptide, this peptide is going to be recognized by cytotoxic Natural Killer (NK) cells (Guzhova & Margulis, 2016). As proven by mice bearing human positive tumor cells, NK cells are important for tumor recognition and eradication (Specht et al., 2015).

HSP70 can also be located extracellularly, where it performs strong immunogenic functions, based on its interactions with the immune system (Nahleh et al., 2012). For example, HSP70 can be transported to the exterior of cancer cells, where it presents certain tumor antigens to Antigen Presenting Cells (APC) (Murshid, Gong, & Calderwood, 2008).

Both interactions mentioned above are the base of anticancer vaccine formulations (Guzhova et al., 2016). In them HSP70 has been administered as an exogenous protein, taking advantage of either its extracellular location or its TKD domain in the cell surface instead (Guzhova et al., 2016). After injection of HSP70 in tumors, this protein is capable of sensitizing cytotoxic T lymphocytes that will later kill the

tumor (Murshid, Gong, Stevenson, & Calderwood, 2011). This innovative therapeutic has shown a lot of potential in *in vivo* animal models (M. A. Shevtsov et al., 2014). One study, for example, injected HSP70 inside the brain of a rat with intracranial tumor, causing a significant delay in tumor growth and increased the survival of tumor bearing animals (M. A. Shevtsov et al., 2014).

HSP90

HSP90 is the most abundant HSP, being present in all eukaryotic cells (Sreedhar, Kalmár, Csermely, & Shen, 2004). It is a constitutively expressed chaperone, representing 1 to 2% of the total protein of a normal cell (Hoter, El-Sabban, & Naim, 2018). These basal levels are what allow organisms to overcome environmental stresses (Taipale, Jarosz, & Lindquist, 2010; Whitesell & Lindquist, 2005). However, following stimulation, it can rise between 4 to 6% (X. Wang et al., 2014).

HSP90 has four major isoforms (Kumar et al., 2017). Firstly, HSP90A, the cytosolic form, is subdivided into HSP90α (the heat inducible) and HSP90β (the constitutively expressed) (Sreedhar et al., 2004). Then, there is the HSP90B1, also called Grp94, which is located in the endoplasmatic reticulum, and Trap, the mitochondrial form (Kumar et al., 2017).

As illustrated in Figure 5, the HSP90 structure consists of three major regions: the N- and C-terminal domains located at both ends and the middle region in between (Hoter et al., 2018; Miyata, Nakamoto, & Neckers, 2013). The N-terminal domain contains the ATP binding site, being responsible for the HSP90 ATPase activity (Hoter et al., 2018). The middle region is where co-chaperones and client proteins will bind (Jackson, 2013). At last, the C-terminal domain has the Tetratricopeptide Repeat (TPR) domain (Brinker et al., 2002). It is where co-chaperones that have this region, such as Hop, bind (Schopf et al., 2017). A general representation of all the domains on HSP90 is presented in Figure 5.

Figure 5 - HSP90 structure. Represented in blue the N- and the C-variable terminal domains and represented in orange the Middle domain. Represented in green there is the TPR Domain. Adapted from Jackson, 2013.

In bovines were found the exact same four HSP90 isoforms discovered in all other species (Ajayi et al., 2018). In cattle the N- and C- terminal regions are also responsible for crucial roles in chaperone activities (Ajayi et al., 2018). Interestingly, it was also discovered that, in cows, the mitochondrial isoform Trap was the first to diverge, along the evolutionary process (Ajayi et al., 2018). This is consistent with

the fact that its primary functions are in the mitochondria, while the others are mainly located in the cytosol (Ajayi et al., 2018).

HSP90 is part of a multichaperone complex and has been identified in several pathways (Nahleh et al., 2012). It is in charge of more than 200 client proteins, that cover almost all cellular processes (Jhaveri & Modi, 2012; Nahleh et al., 2012; Trepel, Mollapour, Giaccone, & Neckers, 2010).These proteins rely on the interaction with HSP90 to become mature, active and stable (McCleese et al., 2009). Some examples are the tumor suppressor gene p53 or the oncoprotein receptor tyrosine kinase ErbB2 (Schopf et al., 2017). All of the important cellular functions in which HSP90 is involved are supervised by more then 20 co-chaperones, such as HSP40 (Sterrenberg et al., 2011; Taipale et al., 2010).

HSP90 is overexpressed in malignant cells, being responsible for the majority of the pathways that lead to cancer and cancer cell metabolism (Miyata et al., 2013; Schopf et al., 2017).

High levels of HSP90 were identified in canine malignant mammary tumors (Romanucci et al., 2006). Although it was also found in normal tissues, its expression is mainly associated with cancer, being related to malignant processes, such as the development of metastasis (Romanucci et al., 2006).

A recent essay found that HSP90, together with some apoptotic proteins, change their pattern of expression in mammary tumors, of both humans and canines (Kumaraguruparan et al., 2006). For example, proapoptotic proteins were significantly decreased, whereas HSP90 and other antiapoptotic members were significantly increased (Kumaraguruparan et al., 2006). This shift of balance towards HSP90 expression confirms, in the first place, that this HSP is really involved in the inhibition of apoptosis and, thus in the expression of malignant properties, like prolonging tumor cell survival (Kumaraguruparan et al., 2006). Secondly, this study highlights the molecular similarities between human and canine mammary neoplasms, suggesting that they can share the same mechanisms to evade apoptosis (Kumaraguruparan et al., 2006).

Grp94, the Endoplasmatic Reticulum homologous of HSP90, has also been reported in canine mammary tumors (Kumar et al., 2017). Grp94 is active during carcinogenesis, because the vast majority of misfolded or unfolded proteins that result from this situation will accumulate in the endoplasmatic reticulum (B. X. Wu, Hong, Zhang, Ansa-addo, & Li, 2016). However, retention of Grp94 in the endoplasmatic reticulum is not absolute, since it has been detected in the plasma of cancer patients (Roveri, Zaccarin, Pagetta, Tramentozzi, & Finotti, 2015). After quantification of circulating levels of Grp94 it was concluded that this protein is overexpressed in dogs with neoplastic mammary glands (Kumar et al., 2017). Likewise, its levels are consistently higher in more complex carcinomas, when compared with simpler benign tumors (Kumar et al., 2017). These findings suggest that, not only Grp94 is involved in mammary tumors, but also prove that it is a valuable biomarker in the diagnosis, prognosis and evolution of the disease (Kumar et al., 2017).

Not only have HSPs been linked to cancer, but some of its client proteins have also been reported as important members of tumor development (Matsuyama et al., 2001). ErbB2, for example, is a HSP90

client protein that is expressed on a large number of neoplasms (Matsuyama et al., 2001). Although ErbB2 is not found in normal mammary tissues, it can be overexpressed in mammary neoplasms of both humans and canines, suggesting its involvement in the tumorigenesis of the mammary gland (Vail & Macewen, 2000).

HER-2 is another HSP90 client protein that is also present in canine mammary carcinomas (Martín de las Mulas, Ordás, Millán, Fernández-Soria, & Ramón y Cajal, 2003). This oncoprotein was exclusive of malignant tumors, being associated with invasive growth, poor prognosis and lower life expectancy (Martín de las Mulas et al., 2003).The percentage of mammary neoplasms with HER-2 overexpression is 17.6%, a value similar to the one reported in human breast cancer (Martín de las Mulas et al., 2003).

In feline mammary carcinomas, HER-2 overexpression is once more related to more aggressive clinical phenotypes (Marques, Correia, & Ferreira, 2016). Its incidence in cats was found to be 20 to 40%, a value also similar to the one found in human patients, suggesting that the feline model can be a suitable model to study HER2 positive human breast cancer (Marques et al., 2016).

Another research discovered that the tumor suppressor gene p53 is another oncogene that plays a strong role in the progression and differentiation of mammary neoplasms (Romanucci et al., 2008).

All of this information regarding HSP90 in canine mammary tumors led to the appearance of the canine model as the best suitable animal model for studying human breast cancer (Kumaraguruparan et al., 2006). It even overtakes the classical rodent model (Kumaraguruparan et al., 2006). However, the feline mammary carcinoma also constitutes a good translational model, since it resembles the more aggressive breast cancer types (Marques et al., 2016). Due to the similarities between species, findings from spontaneous animal mammary tumors can be extend to the human counterpart (Marques et al., 2016; Romanucci et al., 2008).

However, HSP90 was found not only in dog mammary tumors, but also in cutaneous SCC (Romanucci et al., 2008). In both tumors, the shock proteins are located in the centrosome of mitotic cells, controlling metaphase-anaphase transition (Romanucci et al., 2008; Romanucci et al., 2006). The involvement of HSP90 as a regulator of mitotic pathways proves its interference in tumor proliferation (Romanucci et al., 2006).

In bone cancer, HSP90 was one of the most abundant proteins detected in canine appendicular osteosarcoma, where it is envolved in tumor pathogenesis, as well as in the formation of neoplastic emboli (Romanucci et al., 2012). In particular, HSP90α, the inducible form, is associated with poor survival rates (Selvarajah et al., 2013). However, this HSP is largely expressed in all tumor grades, making it impossible to establish a prognostic value (Romanucci et al., 2012). The canine osteosarcoma is considered one of the dog tumors that has the biggest and most impressive similarities with the human counterpart (Romanucci et al., 2012). This way, spontaneous dog osteosarcoma is normally used as a model for the human disease, with several clinical trials undergoing evaluation in animal models (McCleese et al., 2009). HSP90 functions and roles in neoplasms are mentioned in Table 4.

Table 4 - Main characteristics of HSP90 and its functions in both normal and neoplasms.

HSP90					
Functions	**Situation**	**Tumor types**	**Species**	**Tumor Origin**	**Importance in Oncology**
-Protein folding; - Client protein maturation; - Cell growth; - Degradation of misfolded proteins (Wang et al., 2014)	Physiological				
- Inhibition of apoptosis; (Nahleh et al., 2012)	Physiological / Pathological				
- Interfering with mitosis (Romanucci et al., 2008); - Angiogenesis; - Tumor invasion; - Metastasis (Miyata et al., 2013)	Pathological	Mammary Gland Carcinoma	Canines (Kumar et al., 2017; Kumaraguruparan et al., 2006; Romanucci et al., 2006)	Spontaneous (Necropsy + Surgical + Biopsy Samples)	- Role in carcinogenesis and metastasis; - Indicative of poor prognosis -Biomarker for the diagnosis and prognosis of the disease
		Osteosarcoma	Canines (Romanucci et al., 2012; Selvarajah et al., 2013)	Spontaneous (Surgical Samples)	Role in carcinogenesis and metastasis

The intense detection of HSP90 in several canine tumors, suggests that this protein could be used as a potential molecular target in future treatments (Jhaveri & Modi, 2012). To test if its inhibition can affect tumour cells, *in vivo* models became necessary (Romanucci et al., 2006).

HSP90 is the perfect drug target for cancer therapy, among all HSPs (Nahleh et al., 2012). It offers the unique advantage of depleting multiple oncoproteins simultaneously, while attacking different pathways of tumor progression at the same time (Jhaveri & Modi, 2012; Whitesell & Lindquist, 2005). Inhibitors are tested as single agents or in combination with other drugs, however, there is some evidence that

they are less effective and carry more side effects in monotherapy (M. Shevtsov et al., 2019). Their role is to combine them with other molecules (M. Shevtsov et al., 2019).

HSP90 inhibitors have a strong selectivity for cancer cells, compared to normal tissues (Schopf et al., 2017; Whitesell & Lindquist, 2005). This preference is related with the activated conformation that HSP90 acquires in tumor cells (Kamal e al., 2003). HSP90, in cancer, is entirely present in the form of multichaperone complexes, with high ATPase activity, that chaperone oncoproteins (Kamal e al., 2003). The end result is the malignant progression (Kamal e al., 2003). By contrast, HSP90 in normal tissues is present in a latent, uncomplexed state (Kamal e al., 2003).

Targeting HSP90 as a strategy for cancer treatment started with the antitumoral antibiotic, Geldanamycin (Chatterjee & Burns, 2017; Scieglinska et al., 2019). Like most HSP90 natural inhibitors, it binds to the ATPase domain (McCleese et al., 2009). This molecule disrupts the ATP-client protein complex, disabling HSP90 from performing its chaperone functions (Jhaveri & Modi, 2012). In the same way, cancer cells show a higher affinity for Geldanamycin (Sterrenberg et al., 2011).This information suggests the possibility of Geldanamycin being a specific inhibitor of neoplastic tissues, increasing the interest in this drug (Sterrenberg et al., 2011). Geldanamycin proved its potential by inhibiting the motility of rhabdomyosarcoma cells into the bone marrow of mice (Lesko et al., 2007). Unfortunately, its promising potential was limited by its side effects, since it was too toxic to be used in the clinic (Eiseman et al., 2005; Hanson & Vesole, 2009).

The 17-allylamino-17-desmethoxygeldanamycin (also called 17-AAG or tanespimycin) is a synthetic derivative of geldanamycin that possesses strong anticancer activity, in both hematologic and solid tumors (Chatterjee & Burns, 2017; Sydor et al., 2006).The strong antitumor activity of 17-AAG may be related to the formation of the HSP90 super-chaperone complexes (Kamal et al., 2003). This was proven using mouse malignant tissues, where 17-AAG had a bigger affinity for HSP90, compared with normal tissues (Kamal et al., 2003). It acts by dysrupting HSP90 client proteins and their transduction pathways (Solit et al., 2002). For example, in mice bearing human prostate cancer, 17-AAG was associated with a decrease in HSP90 client proteins, like HER-2 (Solit et al., 2002). Although 17-AAG was the first HSP90 inhibitor to enter into a clinical trial, research had to be postpone because of its associated side effects, like poor water solubility and lack of oral bioavailability (Nahleh et al., 2012). Although insolubility could easily be solved with the addition of an excipient, the problem is that some excipients may have their own side effects, causing even more problems (Hanson & Vesole, 2009; Sydor et al., 2006). In the same way, 17-AAG can also be metabolized into potentially toxic metabolites, leading to hepatotoxicity (Eiseman et al., 2005; Solit et al., 2002).

17-dimethylaminonoethylamino-17-demethoxygeldanamycin hydrochloride (also called 17-DMAG or alvespimycin) is another 17-AAG analog (Chatterjee & Burns, 2017). When it was created, it was in an attempt to overcome the poor solubility and toxicities of the first, but keeping the same antitumor effect (Jackson, 2013; Jhaveri & Modi, 2012). Due to its optimization and increased potential, this molecule has been included in a variety of clinical trials (Nahleh et al., 2012).

In one essay, using mice bearing human breast cancer xenografts, 17-DMAG pharmacokinetics and pharmacodynamics were studied (Eiseman et al., 2005). It was concluded that the pharmacokinetics of 17-DMAG in tumor-bearing mice, is very similar to the one found in normal animals: after IV administration, it rapidly decreases in the blood stream, flowing to the tissues (Eiseman et al., 2005). Liver and kidneys are two of the organs where 17-DMAG accumulates preferentially, even more than in tumor tissues, which can be related to its highly recognized hepatotoxicity (Eiseman et al., 2005). On the other hand, the pharmacodynamics of 17-DMAG is related to a significant decrease of HSP90 in neoplastic cells (Eiseman et al., 2005). Surprisingly, despite 17-DMAG being directly targeted to HSP90, it can also interfere with other HSPs, like HSP70, whose levels also decresed in tumors (Eiseman et al., 2005).

When 17-DMAG was about to be approved, all its possible side effects were discovered in preclinical toxicity studies, using laboratory mice and beagle dogs (Glaze et al., 2005). One of the side effects is the hepatotoxicity (Glaze et al., 2005). Even the adjacent gallbladder suffered from inflammation and hemorrhage (Glaze et al., 2005). Kidneys, together with the gastrointestinal tract, were two other major targets for 17-DMAG (Glaze et al., 2005). Similar to dogs, mice also showed hepatic changes and bone marrow atrophy, however, in murine species, the cecum was the most affected organ (Glaze et al., 2005).

IPI-504 (also known as retaspimycin hydrochloride) is another 17-AAG analog (Lianos et al., 2015). This novel molecule is a product of oxidation-reduction reactions, in which the 17-AAG is enzymatically reduced to the hidroquinone form of IPI-504 (Sydor et al., 2006). IPI-504, with its innovative structure, is able to establish tighter interactions with the binding pocket of HSP90, being a more potent inhibitor than 17-AAG (Jhaveri & Modi, 2012). In the same way, IPI-504 also has improved pharmacological properties, like being highly soluble in water (Hanson & Vesole, 2009). This means that it does not need any solubilizing agent, allowing a more favorable administration in the clinic (Hanson & Vesole, 2009). On the pharmacokinetics of IPI-504, it accumulates preferentially in tumor cells, due to, on the one hand, the high levels of HSP90 in cancer cells, but also due to the high affinity that this inhibitor has for the HSP90 in tumor cells (Hanson & Vesole, 2009).

One essay, using the murine animal model, tested IPI-504 in human Multiple Myeloma (MM) (Sydor et al., 2006). As a single agent, IPI-504 was capable of delaying tumor progression, however the greatest benefits of this molecule were only discovered when it was combined with bortezomib, a proteasome inhibitor (Sydor et al., 2006). The combination of both is much more effective than using the two agents separately (Sydor et al., 2006). The synergistic potential can be explained by the disruption of protein homeostasis, since IPI-504 destabilizes HSP90 client proteins and bortezomib inhibits their degradation (Sydor et al., 2006).

STA-9090 belongs to a class of HSP90 inhibitors that is unrelated to geldanamycin, being classified as a Radicicol derivative (Y. Wang, Trepel, Neckers, & Giaccone, 2010). STA-9090 is an active metabolite of another HSP90 inhibitor, the STA-1474, which is administered as a prodrug (McCleese et al., 2009).

This means that the metabolization of STA-1474 will originate the STA-9090 (McCleese et al., 2009). The efficacy of STA-1474 was evaluated in mice bearing canine osteosarcoma xenografts: female mice were subcutaneously injected with a canine osteosarcoma cell line and later treated with I.V. injections of STA-1474 (McCleese et al., 2009). This drug not only promoted apoptosis and disruption of HSP90 client proteins, but also induced tumor regression (McCleese et al., 2009). This data suggests that HSP90 can be an important target in the treatment of osteosarcoma, not only in dogs, but also in humans (McCleese et al., 2009).

Another problem is the acquired resistance that HSP90 N-domain inhibitors develop (Jhaveri & Modi, 2012). Pharmacological inhibition of HSP90, through the activation of the Heat Shock Response, leads to the consequent increase of HSP27 and HSP70 (Nahleh et al., 2012). However, both HSP27 and HSP70 have antiapoptotic properties and tumorigenic action (X. Wang et al., 2014). Under these circumstances, the use of HSP90 inhibitors may have the opposite effect and impair cancer treatment (X. Wang et al., 2014). Furthermore, it was hypothesized that the development of HSP70 inhibitors that target HSP90 simultaneously, will offer therapeutic advantages (Murphy, 2013; M. Shevtsov et al., 2019). The HSP90 inhibitors, together with their use in animal models, are reported in Table 5.

Animal models for HSPs investigation

Translation of a therapy into the clinic requires evidence of its efficacy and safety first (Romanucci et al., 2006). That is why animal models are so important in the development of HSPs inhibitors. Since all molecules described previously are still in the development phase, experiments like this, which allow a drug to be first administered in animals, are the only way of ensuring the safety of future human patients (Talmadge et al., 2007).

Most investigations use preferably laboratory rodents or beagle dogs (Vail & Macewen, 2000). However, tumors of companion animals constitute the best way to study human cancers and their experimental therapeutics (Marques et al., 2016; Vail & Macewen, 2000).

Table 5 - HSP90 inhibitors, with special emphasis on their application in animal models.

HSP90 inhibitor	Animal Model	Cell Lines	Administration of Cell lines	Biological Sample	Oncologic Importance
Geldanamycin (Lesko et al., 2007)	Mice	Human Rhabdo myosarcoma cell lines (SMS-CTR + RH30)	Injected supraorbitally	Bone marrow cells	Inhibits seeding into bone marrow
117-AAG (Solit et al., 2002)	Mice bearing human cancer xenografts	Human Prostate cancer cell lines (CWR22 + CWR22R + CWRSA6)	Inoculated subcutaneously	Cells removed from the xenograft	Reduction of HER2 expression
17-DMAG (Eiseman et al., 2005; Glaze et al., 2005)	Mice bearing human cancer xenografts	Human Breast cancer cell lines (MDA-MB-231)	Inoculated subcutaneously on the right flank	Blood at tissue collection at necropsy	Pharmacokinetics and Pharmacodynamics studies
	Rats and Beagle dogs	---	---	Blood at tissue collection at necropsy	Preclinical toxicity studies
IPI-504 (Sydor et al., 2006)	Mice	---	---	Blood collection	IPI-504 results from the oxidation of 17-AAG
	Mice bearing human cancer xenografts	Human Multiple Myeloma cell lines (RPMI-8226)	Inoculated subcutaneously on the right back leg	Blood at tissue collection at necropsy	- IPI-504 inhibits tumor growth alone; - IPI-504 should be combined with bortezomib for better results.
STA-1474 (McCleese et al., 2009).	Mice bearing human cancer xenografts	Canine Osteosarcoma cell lines (D17)	Inoculated subcutaneously on the flank	Cells removed from the xenograft	STA-1474 inhibits tumor growth

Canine and feline cancers are more similar to human tumors in terms of body size or cell biology, providing a great way to investigate many aspects of malignancy, since carcinogenesis is very similar in the two species (Marques et al., 2016). These species share a lot of epidemiological, morphologic and biological features, regarding tumor progression and response to therapy (Romanucci et al., 2008).

In the same way, dogs, as companian animals, live within the same household as humans, being exposed to the same environmental risks (Tarone et al., 2019). More specifically, spontaneous canine tumors, because of their natural occurrence, appear as the best animal model for human neoplasms (Romanucci et al., 2008).

Conclusions

Given the intricate pathophysiology, low prognostic and drug resistance, cancer still remains a poorly understood medical condition. The clinical sciences are in great need of innovative researches that provide insightful informations about oncologic mechanisms and safe therapeutic approaches. The majority of tumors can have a good outcome, if diagnosed early. However, at present, there is a need to develop less invasive methods, which still allow an early diagnosis.

In the last decades, Translational Oncology has become a very important field. It provides a new way of accessing neplasms, based on a molecular prespective. Tumor pathways are followed at a microscopic level, with nanoproteins arising as potential anticancer targets.

HSPs are examples of proteins with therapeutic application. They have the potential to become biomarkers of carcinogenesis, in both human and animals. Due to their involvement in tumor survival, their pharmacological inhibition is seen as a new approach in cancer treatment. There are already several drug molecules that have been, or still are under clinical trials, specially the ones targeting HSP27, HSP70 or HSP90. Despite the fact that some cause adverse effects, HSPs inhibition is still in the early stages of development and carry promising results.

Veterinary medicine is essential in the creation and development of new drugs, since animal models are the basis for clinical trials. However, another interesting advantage of using dogs in preclinical studies is that, when these new molecules eventually transit to the domestic animal clinic, dogs will become patients and not experimental animals. In that situation, all the adverse side effects that can happen in dogs will be already available for clinicians to know, because they have been studied already. This way, using companion animals while seeking innovative and promising therapies offers the unique advantage of treating cancer in canines and human beings at the same time.

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