UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CAMPUS DE JABOTICABAL

EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DEACETYLASES 1, 2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

Oscar Rodrigo Sierra Matiz

Médico Veterinário

2019

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Oscar Rodrigo Sierra Matiz

Orientadora: Prof. Dra. Mirela Tinucci Costa

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Doutor em Medicina Veterinária, área de Clínica Médica Veterinária.

2019

M433e

Matiz, Oscar Rodrigo Sierra
Evaluation of acetylated histones 3 and 4 and histone deacetylases 1, 2 and 6 in cutaneous T-cell lymphoma in dogs / Oscar Rodrigo Sierra Matiz. -- Jaboticabal, 2019
81 p. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientadora: Mirela Tinucci Costa

1. Medicina veterinária. 2. Linfoma. 3. Epigenética. 4. Enzimas. 5. Western blotting. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DEACETYLASES 1,2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

AUTOR: OSCAR RODRIGO SIERRA MATIZ
ORIENTADORA: MIRELA TINUCCI COSTA

Aprovado como parte das exigências para obtenção do Título de Doutor em MEDICINA VETERINÁRIA, área: Clínica Médica Veterinária pela Comissão Examinadora:

Profa. Dra. MIRELA TINUCCI COSTA
Departamento de Clínica e Cirurgia Veterinária / FCAV - UNESP - Jaboticabal

Profa. Dra. JULIETA RODINI ENGRACIA DE MORAES
Departamento de Patologia Veterinária / FCAV / UNESP - Jaboticabal

Prof. Dr. HENRIQUE CÉSAR SANTEJO SILVEIRA
Centro de Pesquisa em Oncologia Molecular. / Hospital de Câncer de Barretos/SP

Pesquisador Dr. FELIPE AUGUSTO RUIZ SUEIRO
Laboratório VetPat / Campinas/SP

Prof. Dr. PAULO CÉSAR JARK
UNICASTELO / Descalvado/SP

Jaboticabal, 25 de novembro de 2019

ABOUT THE AUTHOR

OSCAR RODRIGO SIERRA MATIZ –born in Bogotá, Colombia, on april the 15th of 1987, child of Antonio José Sierra Caviedes and Ruth Jesus Matiz Rodriguez. He studied Veterinary Medicine in La Salle University, Bogotá, Colombia and took his degree in December 2010 under the supervision of Dr. Oscar Javier Benavides. He worked as a resident of internal medicine at the Dover Veterinary Clinic in Bogotá, Colombia from 2011 to 2013. He performed international externships in the oncology department of *Madison-Wisconsin University*, under the supervision of Dr. David Vail in 2013, in the *Coral Springs Animal Hospital*, under supervision of Dr. Francisco Alvarez in 2014 and in *Purdue University*, under supervision of Dr. Michael O. Childress, in 2015. He started his Master of Science (MSc.) studies in 2014 in the Faculty of Agricultural and Veterinary Sciences under the Graduate Program of Veterinary Medicine, area of Veterinary Medical Clinics of the São Paulo State University – UNESP, Campus of Jaboticabal under the supervision of Profa. Dra. Sabryna Gouveia Calazans. In March 2016, he started his Doctoral studies (PhD.) under the same Graduation Program at the same institution under the supervision of Profa. Dra. Mirela Tinucci Costa. During his Postgraduate studies, he was elected as a monitor in the *lato sensu* Posgraduate course of Veterinary Oncology in the Bioethicus Institute, Botucatu, SP from 2014 to 2016 and later, from 2017 to 2018, in the 2nd Course of Oncologic and Reconstructive Veterinary Surgery at the São Paulo State University – UNESP, Campus of Jaboticabal. Since 2014 until now he is attending as a voluntary in the Veterinary Oncology Service (SOV) of the Veterinary Hospital “Governador Laudo Natel” and has published several articles and scientific abstracts in international and national journals and annals of congress, as well as has participated as an author in the oncology section of book chapters including “Oncologia em cães e gatos”, “Medicina Felina Essencial” and “Dia a Dia” (published in Portuguese). Sierra Matiz has been supported by the Coordination for the Improvement of Higher Education Personnel – CAPES Foundation during his Posgraduate studies at the UNESP – Campus of Jaboticabal.

“La vida no es lo que uno vivió, sino la que uno recuerda y cómo la recuerda para contarla”

Gabriel García Márquez

À memória de Antonio e Elkin, e aos meus anjos nesta terra Ruth e Sandra

A dedico

ACKNOWLEDGEMENTS

I do believe there is one God who has seen me with good eyes and a tender look. During these years I have been through difficult situations, but when I look back I can see myself being covered by his gentile and warm embrace. To Him, ***gracias!***

I am what I am because of my father and mother. My father Antonio left me in the middle of my doctoral course, and since he is not anymore in this earth, I remember his presence and advices day by day, and follow his good manners and behavior, trying to honor his name in my simple days. My mother Ruth is more than just my mum, she has become my best friend, my rock and my safe harbor. When I am with her I do not feel any pain or if I felt it, it would be rapidly diminished. I want to thank my father because I am a better person because of his teachings and example of life. And I want to thank my mother because she makes my life easier and happier, she is my best gift in this world, my mentor and angel in this earth. To them, ***gracias!***

My childhood partners and friends of life are my brother Elkin and my sister Sandra. Elkincito left us at the end of my masters studies and his absence has dug very deeply inside my heart. I want to thank him because first of all, he taught me how to see happiness in the simple details of life and second of all, his absence has brought the most difficult test to overcome. It is possible that we as a family needed to go through this almost unbeatable test to encourage ourselves in the most difficult moments, and he taught us this, to stay closer and to be stronger. My sister is my walking stick. Sandrita has been my example of love and support, not only for me but for her family. My nephews have a competent mother and I want to thank her because she is present at any moment, she is unconditional. To my brother and sister, ***gracias!***

My life in Brazil has been enjoyable and easy since I am surrounded by the right people. I want to thank Brazil for embracing me since the beginning of my life here and to my friends in this small and beauty town. Specially, I want to thank all my Brazilian friends Paulo, Denner, Juliana, Roana, Delvana, Celso, Nazilton and my Latin-American friends Jorge, Noelia, Nathalia, Brayan, Ricardo, Diego Rojas, Diego Rodriguez, Juan Diego, Juan Esteban, Estefania and Pablo. To my friends that stay

with me in the distance and that became my brothers in Colombia, Diego, Jose, John, Andrés Julian, Juan Camilo, Ricardo, Cindy, Carla and Andrea. To all of them, ***gracias!***

One person knows about all the effort I put in this study, she has been unconditional and more than my love, Natalia is my friend, scientific colleague and partner. I want to thank Natalia because I can rely on her, she has taught me to continue walking no matter what we face. To her, ***gracias!***

One door was opened when profa. Mirela accepted me as her student. This project was impossible to execute without her help and support. She is my mentor and example of teacher; her charisma inspires me. And my friends of lab, Igor and Isabela, who stay with me and together formed a team who overcame scientific obstacles. To all of them, ***gracias!***

I want to thank the oncology service of UNESP Jaboticabal, the great SOV, all of my colleagues have impacted with their teachings my knowledge of oncology and life, specially, Stella, Milena, Rafaela, Gabriel, Marla, Gabriela, Pedro, Thuanny and prof. Andrigo, who his support was fundamental to be at this stage. To all of them, ***gracias!***

And last but not least, to all the dogs and cats I found in this journey, they taught me how to be an oncologist and more important, a better veterinarian. To all of them,

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, ***gracias!***

SUMMARY

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CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Avaliação da acetilação e da expressão das enzimas HDAC1, HDAC2 e HDAC6 como mecanismo epigenetico em linfoma cutâneo de cães"**, protocolo nº 019049/17, sob a responsabilidade da Prof.ª Dr.ª Sabryna Gouveia Calazans, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 07 de dezembro de 2017.

Vigência do Projeto	12/12/2017 a 01/12/2019
Espécie / Linhagem	*Canis lúpus familiaris*
Nº de animais	32
Peso / Idade	Aleatório
Sexo	Ambos os sexos
Origem	Atendimento do Hospital Veterinário "Governador Laudo Natel" UNESP Jaboticabal

Jaboticabal, 07 de dezembro de 2017.

Prof. Dr. Everlon Cid Rigobelo
Vice Coordenador – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n - 14884-900 - Jaboticabal - SP - Brasil
tel 16 3209 2600 fax 3202 4275 www.fcav.unesp.br

AVALIAÇÃO DAS HISTONAS ACETILADAS 3 E 4 E DAS HISTONAS DESACETILASES 1, 2 E 6 EM LINFOMA CUTÂNEO DE CÉLULAS T EM CÃES

RESUMO- O linfoma cutâneo constitui uma forma de linfoma extranodal que afeta inicialmente a pele e/ou anexos cutâneos e que pode apresentar um curso clínico agressivo, avançando a órgãos internos em estádios tardios. O principal imunofenótipo é de células T (LCCT) e representa a forma mais comumente diagnosticada. É esperado que os cães com LCCT desenvolvam resistência à quimioterapia em alguma etapa do tratamento, resultando em tempos curtos de sobrevida. Há uma necessidade por novos alvos de tratamento em cães com LCCT que consigam fornecer melhor expectativa de vida. Em humanos, medicações baseadas em mecanismos epigenéticos têm auxiliado no controle da doença sendo um alvo chave na procura de repostas clínicas mais douradoras em estádios avançados. Neste trabalho descreve-se, nos dois primeiros capítulos relatados, o LCCT e as particularidades descritas na literatura internacional que contrasta com os dados encontrados nesta instituição, assim como, o papel da epigenética na carcinogênese das neoplasias e o mecanismo de modificação de histonas como base para o tratamento. Mais especificamente, no segundo capítulo, foram analisados dados de 21 cães com linfoma cutâneo definido como LCCT de alto grau devido ao tamanho grande das células, à ausência de tropismo na epiderme, ao valor médio encontrado de Ki67 de 63,9% e ao tempo de sobrevida estimado de 31 dias. Características clinico-patológicas foram analisadas para a identificação de marcadores prognóstico, sendo definido as alterações decorrentes pelo linfoma vistas na radiografia como marcador prognóstico negativo independente depois da análise multivariada. No último capítulo relatado, amostras de LCCT foram avaliadas para conhecer o nível de histonas acetiladas (H3Ac e H4Ac) e de histonas deacetilases (HDAC) HDAC1, HDAC2 e HDAC6, as quais encontram-se envolvidas no mecanismo de modificação de histonas em LCCT de humanos. O objetivo desse estudo foi comparar amostras de LCCT com linfonodo normal, pele inflamada e pele normal de cães. Tanto as H3Ac e H4Ac como as HDAC1, HDAC2 e HDAC6 estiveram aumentadas em LCCT quando comparadas com pele normal, de igual maneira encontrou-se que o nível de H3Ac foi estatisticamente menor em linfoma que em linfonodo normal, e que um aumento aberrante de H4Ac e HDAC2 foi constatado em LCCT. Adicionalmente, evidenciou-se que a associação da imunoexpressão de H3Ac, H4Ac e HDAC2 classificou duas populações, as quais apresentaram tempos de sobrevida diferentes (48 dias Vs 22 dias, p=0.06), sugerindo assim um perfil de histonas existente em amostras de LCCT. Este estudo confirmou que o nível de histonas deacetilases em LCCT é maior que em tecidos saudáveis. Futuros estudos são necessários para corroborar nossos resultados e para que futuramente inibidores das HDAC possam ser utilizados em cães com LCCT.

Palavras-chave: acetilação, canino, epigenética, HDAC, linfoma extranodal, oncologia

EVALUATION OF ACETYLATED HISTONES 3 AND 4 AND HISTONE DESACETYLASE 1, 2 AND 6 IN CUTANEOUS T-CELL LYMPHOMA IN DOGS

ABSTRACT- Cutaneous lymphoma constitutes a form of extranodal lymphoma that affects initially the skin and/or adnexal structures and eventually presents an aggressive clinical behavior, causing internal organ infiltration in late-stage disease. The main immunophenotype is T-cell lymphoma (CTCL) and it represents the most common diagnosed type. It is expected that dogs with CTCL develop resistance to chemotherapy at any point during treatment, resulting in short survival times. A need for new therapeutic targets that improve survival expectation in dogs with CTCL is increasing. In humans, therapies based on epigenetic mechanisms helped to control the disease, since epigenetics became a main objective in the search for longer clinical responses in advance-stages. In the first two reported chapters, CTCL is described with particularities of international literature that contrast with data found in this institution, as well as, the role of epigenetics in the neoplasia carcinogenesis and the mechanism of histone modification as a base for treatment. More specifically, in the second chapter, 21 dogs with CTCL were defined as having high grade CTCL due to their large cell size, absence of epithelial tropism, mean value of Ki67 of 63,9% and estimated survival time of 31 days. Clinicopathological characteristics were analyzed for identifying prognostic markers, being the intrathoracic involvement caused by lymphoma seen on thoracic radiography an independent prognostic factor after multivariate analysis. In the last study, samples of CTCL were evaluated to know the level of acetylated histones (H3Ac and H4Ac) and histone deacetylate enzymes (HDAC) HDAC1, HDAC2 and HDAC6, which were involved in the epigenetic mechanism of histone modification in human CTCL. The objective of this study was to compare samples of CTCL in normal lymphnode, inflammatory cells in skin and normal epithelial cells. All markers (H3Ac, H4Ac, HDAC1, HDAC2 and HDAC6) were found to be higher in CTCL than in normal skin, furthermore, the level of H3Ac was statistically lower in CTCL than in normal lymphnode and an aberrant higher level of H4Ac and HDAC2 was confirmed in CTCL. Additionally, the association of the immunoexpression of H3Ac, H4Ac and HDAC2 classified into two the population, having different survival times (48 days Vs 22 days, p=0.06), suggesting that a histone profile exists in the studied population. This study confirmed that the level of histone deacetylases in CTCL are higher than in normal tissues. Further studies are needed to confirm our results and to support new research in HDAC inhibitors in dogs with CTCL.

Keywords: acetylation, canine, epigenetic, extranodal lymphoma, HDAC, oncology

LIST OF TABLES

Page

CHAPTER 2 - Clinical description and prognostic factors of high grade cutaneous T-cell lymphoma in dogs

CHAPTER 3 - Expression profile of acetylated histones 3 and 4 and histone deacetylate 1, 2 and 6 and their association in cutaneous T-cell lymphoma in dogs

LIST OF FIGURES

Page

CHAPTER 2 - Clinical description and prognostic factors of high grade cutaneous T-cell lymphoma in dogs

CHAPTER 1 - General considerations

1. Introduction

The term lymphoma involves a group of diverse diseases that have a common cell of origin but differs among locations and several other aspects including tumor behavior, clinical manifestation, treatment approach and prognosis. In this regard, cutaneous lymphoma (CL) is considered a rare and incurable form of lymphoma that affects multiple species (Kuzel et al., 1991; Fontaine et al., 2009; Fontaine, Heimann and Day, 2011; Miller et al., 2015). In dogs, CL is characterized by the presence of variable cutaneous signs and challenging treatment that finally lead the patient to disease progression and poor prognosis (Fontaine et al., 2009; Rook, 2019). In Brazil, information about this disease is scarce; incidence seems to be higher and survival times shorter than the reported in international literature (Duarte et al., 2016).

Canine CL has been widely reported to respond to chemotherapy, therefore, different types of drugs have been used against this disease (Lemarie and Eddlestone, 1997; De Loremier, 2006; Risbon et al., 2006; Williams et al., 2006; Morges et al., 2014), but mean of survival time does not exceed 6 months according to a recent treatment review (Laprais and Olivry, 2017). In humans, the classical form of CL is called Mycosis Fungoides (MF) and this disease manifests as patches or plaques at initial steps and nodules or systemic dissemination in late stages (Olsen et al., 2007a). Despite the good prognosis of MF in humans, eventually cases of systemic involvement and multiple nodules develop in different areas of the body and systemic treatments based on chemotherapy are combined with a range of new drugs (Li et al., 2012). One type of drugs used in advanced MF are histone deacetylase (HDAC) inhibitors that are approved by the FDA since 2006 (Olsen et al., 2007b; Li et al., 2012). These kind of drugs promote response through histone acetylation that lead the cells to permit the transcription of suppressor genes, resulting in apoptosis and inhibition of tumor proliferation (Lane and Chabner, 2009; Khan and Thangue, 2012).

The use of HDAC inhibitors is rarely reported in veterinary medicine and it is unknown if a positive effect may be expected in advance stages of CL in dogs, as happens in humans. In order to answer this question, the objective of this research is to know if an acetylation profile exist in dogs with CL that can justify the use of HDAC inhibitors in prospective studies. Additionally, it was imperative to report the findings of a more aggressive CL that is evident in the veterinary hospital of the São Paulo State University, UNESP –Jaboticabal.

2. Literature review

CL constitutes an extra-nodal form of lymphoma characterized by the infiltration of malignant lymphocytes in the epidermis, dermis and adnexal structures (Miller et al., 2013). Other reported forms involve the oral mucosa and mucocutaneous junctions (Fontaine 2010, Chan et al., 2018). CL has a variable clinical presentation and the diversity of cutaneous lesions hinders the diagnosis at initial stages. Based on international literature, CL has a low incidence and is reported as a rare disease (Goldschmidt, 1992; Fontaine et al., 2009). In Brazil, there is no exact information regarding its incidence, but 60 cases were reported in a period of 6 years between 2007 and 2013 in the Veterinary Hospital of UNESP Jaboticabal (Jark et al., 2014), and over 40 cases were diagnosed based on the current caseload at the same institution.

In humans, most of the forms of CL present an indolent behavior and a detailed staging is required to define a more specific treatment and prognosis. Cutaneous T-cell lymphoma (CTCL) represents most of the cases of CL and can be further classify regarding cell size, localization of lymphocytes in epidermis, expression of CD4, CD8 and CD30 and T-cell receptor phenotype (α/β or γ/δ) (Willemze et al., 2019). Human CTCL constitutes a heterogeneous disease having more than ten primary types and variants, according to the most updated classification of this disease in humans (Willemze et al., 2019). The typical form of CTCL, MF was first described by Jean-Louis Alibert in 1806 as a chronic dermatitis presenting a mass formation and a fungal

appearance (Fontaine et al., 2009). MF constitutes the most common form of CL reported in humans and is characterized by the tropism of malignant T-lymphocytes to the epidermis and its adnexal structures (epiteliotropism) (Goldschmidt, 1992). Occasionally, a group of malignant cells gathered in the epidermis forming a Pautrier abscess, a histology characteristic of MF in humans (Willemze et al., 2019). This disease in humans has a chronic course and prognosis is good in early-stages, only advance-stages present invasion of deep layers of the skin and even infiltration of blood and other organs; an aggressive form recognized as Sezary Syndrome (SS) (Willemze et al., 2005).

In contrast, dogs with CL have a more aggressive course of the disease and prognosis seem to be poor (Risbon et al., 2006; Williams et al., 2006; Fontaine et al., 2009; Fontaine et al., 2010). Response to chemotherapy protocols contemplating multiple drugs is limited and last for only two to six months (Williams et al., 2006, Risbon et al., 2006; Morges et al., 2014; Holtermann et al., 2016; Laprais and Olivry, 2017), although a previous report described dogs surviving for up to two years (Fontaine et al., 2009). This difference in survival and prognosis between human and canine lymphoma is still uncertain, but two important molecular characteristics of CL differs from one species to the another: immunophenotype and type of T-cell receptor. Malignant lymphocytes in CL can be classified according to the positive expression of CD3 and/or CD79a. Cutaneous T-cell lymphoma (CTCL) presents a typical CD3+CD79a- phenotype, however a double expression CD3+CD79a+ is also described in up to 30% (Day, 1995; Fontaine et al., 2010; Chan et al., 2018). Cutaneous B-cell lymphoma CD3-CD79a+ (CBCL) seems to be less common since few reports are encountered in veterinary literature (Day, 1995, De Bosschere and Declerq, 2008) and double negative expression CD3-CD79a- has also reported in cases of epitheliotropic CL (Day, 1995). In CTCL, T-lymphocytes may further be classified regarding its positive immunoreactivity to CD4 or CD8 antibodies. Immunophenotype of human T-cells is mainly CD4+CD8-, whereas dogs present a CD4-CD8+ profile (Moore and Olivry, 1994; Moore et al., 2009; Willemze et al., 2019). Also, the T-cell receptor (TCR) phenotype differs between both species. In humans, a TCR-αβ is most commonly expressed (85-90%) while TCR- γδ are only found in few lymphomas (10-15%) that may present a more aggressive course of the disease (Toro

et al., 2003). In contrast, a TCR-γδ is more frequently encountered in the canine counterpart (60%), when compared to TCR- αβ (40%) (Moore et al., 2009). Although these differences may not explain why canine CTCL have a non-indolent clinical course and a shorter survival time when compared to humans, it highlights the importance of a most specific characterization and the need for more studies in CTCL that help to understand better this disease in pets.

2.1 Etiology and immunopathogenesis

Etiology of CTCL is still unknown (Fontaine et al., 2009). In humans, genetic, immunologic and infectious factors have influenced somewhat the development of cutaneous lymphoma, however, literature is inconsistent and results are controversial. Human leukemic T-cell virus was described as an etiologic agent (Pancake, Zucker-Franklin and Coutavas, 1995), but others denied this information (Li et al., 1996). Some other virus like Eipstein—Barr virus and Human Herpesvirus (HHV-8) were found in lymphoproliferative lesions and Hodgkin-lymphoma in humans, but it seems to be an associated factor rather than an etiology cause (Trento et al., 2005; Ahmed and Heslop, 2006). In dogs, no studies have shown an association between an infectious agent and cutaneous lymphoma. Nevertheless, chronic antigenic stimulus that result in clonal expansion of lymphocytes can be an initial tumor event (Willemze, 2003). In dogs, this hypothesis has been studied and some authors found a 12 times increased risk of developing cutaneous lymphoma in dogs with previous history of atopic dermatitis (Santoro et al., 2007). Other authors linked the topographical sites of atopic dermatitis (mucocutaneous junctions and feet) with locations of development of CTCL based on the TCR γδ phenotype and made hypothesis of the traffic of T-cells to these sites secondary to an immune stimulus, demonstrating a possible relationship between inflammation and CL development (Moore et al., 2009). However, results are controversial since Beale and Bolon (1993) found that only 23% of cases had chronic dermatitis before diagnosis of CL and in contrast, Fontaine et al. (2010) found no history of dermatitis in any of the 30 cases diagnosed with CTCL.

Physiologically, T-lymphocytes need to migrate to the skin in response to skin insults to initiate and maintain cutaneous inflammation (Hwang, 2001). In order to do so, T-cells express surface molecules such as CLA and CCR4 that are recognized by endothelial receptors including E-selectin and CCL17 in places where migration from blood to epidermis is required (Reiss et al., 2001). When this happen, skin T-lymphocytes become activated and send signals in conjunction with Langerhans cells through the expression of the ligand CCL22 to recruit more cells (Girardi et al., 2004). In CTCL, ligands like CCL17 and CCL22 are found to be overexpressed and it has been determined that keratinocytes and Langerhans cells enhance the migration of T-lymphocytes by the expression or more molecules including E-cadherin and ICAM-1 (Heald et al., 1993; Ferenczi et al., 2002). A clonal expansion of malignant lymphocytes occurs in the epidermis since detection of clonality has been found in the skin (Girardi et al., 2004). Activation of malignant lymphocytes stimulate the production of several interleukins (IL) including IL-4 and IL-5 that promote inflammation and exacerbate the clinical manifestation (Papadavid et al., 2003). Also, high levels of IL-10 will downregulate the immune response from the host and high amounts of IL-2 receptor will competitively bind the IL-2 necessary for physiological T-cell activation (Bagot et al., 2001). As a result, a biased type 2 immune response is elicited and a more favorable tumor microenvironment is created (Girardi et al., 2004).

2.2 Epidemiology

CL represents between 3-8% of all lymphoma types and about 1% of all cutaneous tumors in dogs (Goldschmidt and Shofer, 1992; Fournel-Fleury 2002). It is recognized as a rare disease and the largest number of dogs with CTCL (148 dogs) was reported by Chan et al., (2018) in Australia during a period of 12 years. The low incidence can also be explained by an underrated number of confirmed diagnosis, since many cases of CL can be misdiagnosed and treated as a dermatitis until a biopsy is performed. For a group of veterinary dermatologists in Europe (Fontaine et al., 2009) the incidence seemed to be higher than the described in literature.

Some studies have found no sexual or breed predisposition (Goldschmidt, 1998; Risbon et al., 2006; Williams et al., 2006), but others found a more predominant number of females, when compared to male dogs (Chan et al., 2018; Ewing et al., 2019). In previous studies the Cocker Spaniel, Boxer and Golden Retriever were overrepresented (Fontaine et al., 2009, Moore et al., 2009; Chan et al., 2018), moreover, a Belgium study indicated also the Bichon fries (Fontaine et al., 2010). In Brazil, mixed-bred dogs seemed to be overrepresented and pure-bred dogs like Poodle, Boxer and Pit Bull were more frequently diagnosed with CL (Jark et al., 2014; Duarte et al., 2016). CTCL affects typically old dogs with median age ranging from 8,6 to 11 years-old (Fontaine et al., 2009; Moore et al., 2009; Fontaine et al., 2010; Chan et al., 2018).

2.3 Clinical signs

The clinical manifestation of patients with CL is highly variable and depends on the type of cutaneous lesion, tumor burden and stage. In humans, the type of lesion (patch, plaques or nodules) and tumor burden (< or >10% of skin covered by lesions) is related to the stage of the disease (Olsen et al., 2007a). Also, clinical signs are dependent on the histological form of CL. More specifically in CTCL, initial lesions in humans are only small lesions in patches or plaques, but nodules of different sizes are evident in advanced disease. In veterinary, Fontaine et al., (2009) classified clinical lesions of dogs with CTCL into four groups: exfolliative erythroderma, patches, plaques or nodules, mucocutaneous lesion and oral mucosa form. Exfolliative erythroderma is recognized as an early-stage of the disease in dogs, whereas in humans constitute a late stage (Fontaine et al., 2009). Recently, Chan et al., (2018) classified CTCL only into two large groups: cutaneous form (including any type of lesion) and mucocutaneous-oral mucosa form.

Multiple nodules are more frequently reported than any other form in canine CTCL and are present in higher amount in the skin than in the mucocutaneous junction and oral mucosa (Chan et al., 2018). An arciform lesion constitute a characteristic seen

in cases of CTCL in humans and dogs (Duncan, 2011; Miller et al., 2013). This lesion is arched or has a shape of a bow and is commonly evident in the Veterinary Hospital of UNESP Jaboticabal. Sometimes they ulcerate and secondary contamination takes places, deteriorating the clinical condition. Nodules of different sizes, patches or plaques can be overlapped and may be seen at any stage of the disease in dogs (Fontaine et al., 2009). Other signs like pruritus, scaling, erythema and depigmentation may accompany the whole clinical image of a dog with CTCL, however, most of them are associated to the exfolliative erythroderma form (Rook, 2019). These signs may be attributable to allergic dermatitis, parasitic infestation of the skin or secondary bacterial infections that make the final diagnosis confusing (Miller et al., 2013).

As disease progresses new nodules emerge from subcutaneous tissue and deep cutaneous infiltration causes edema than finally affects limbs. SS may be encountered in late-stage CTCL in exceptional cases and is recognized by finding lymphocytes with cerebriform nuclei in the peripheral circulation (Rook, 2019). This atypical manifestation is observed in approximately 5% of cases in human CTCL (Hwang et al., 2008).

2.4 Diagnosis

Cases of CTCL can be suspected based on clinical examination. Multiple nodules and the presence of arciform lesions with or without history of previous dermatitis may rise the suspicion. A practical diagnostic approach is made by cytological visualization of malignant lymphocytes from cutaneous nodular lesions, but definitive diagnosis will require histopathological evaluation (Rook, 2019). Skin biopsy taken from representative lesions should be performed. A clear differentiation of malignant from reactive lymphocytes may be problematic in inflammatory processes that presented with exocytosis of lymphocytes. In nodular lesions of the skin, CTCL must be differentiated from other round cell neoplasms including mast cell tumor, histiocytic sarcoma and more rarely, extragenital TVT forms (Fontaine et al., 2009). Histologically, malignant lymphocytes can be classified according to the presence or

absence of tropism to epidermis and adnexal structures, defining it as epitheliotropic and non-epitheliotropic CL, respectively (Goldschmidt and Shofer, 1992). Epitheliotropic CL is also known as CTCL, since epiteliotropism is only found in malignant T-cells, whereas non-epitheliotropic CL can be composed by T or B-cells (Moore and Olivry, 1994; Day, 1995).

Immunophenotyping for CD3 and CD79a is easily established by immunohistochemistry after biopsy results. CD3+CD79a- is expressed in 70-97,5% of cases of CTCL and double positive immunoreactivity can be found in up to 30% of cases (Day, 1995; Fontaine et al., 2010; Chan et al., 2018). Cases of double negative expression have been described in epitheliotropic CL in 42,8% (Day, 1995). CD4 and CD8 expression in CTCL has only been described by immunoexpression of snap frozen samples and flow cytometry (Moore et al., 1994; Moore et al., 2009). Several studies have proved the different CD4-CD8+ immunoreactivity in dogs with CTCL, when compared to the human most common form CD4+CD8- (Moore et al., 1994; Moore et al., 2009; Willemze et al., 2019). Additionally, canine T-cells present the TCR-δγ more commonly reported than the TCR-αβ (Moore et al., 2009).

Clonal expansion of malignant lymphocytes is a common feature from all lymphomas and it has been found in about 80% of T-cells by PCR of the antigen receptor gene rearrangement or by Southern Blot in an older study (Fivenson et al., 1994; Moore et al., 2009). More recently, a study aimed to detect the B-cell marker CD20 in canine CTCL, since co-expression of CD20+ have a worse prognosis in human literature (Julié et al., 2013). CD20+ T-cells were found in 54% of CTCL, but its expression was not associated with shorter survival time (Ewing et al., 2018).

2.5 Treatment

It seems that CTCL in dogs has a more aggressive form than the human counterpart. Although responses with systemic treatments based on chemotherapy are expected, the disease turns irresponsive and cutaneous lesions advance through systemic dissemination or euthanasia is elected by owners as ulceration and infection

progress in the skin (Rook, 2019). Local treatments can be beneficial for a restricted population with solitary masses without systemic spreading of the disease, in this regard, surgery was described as initial treatment for nodules in skin, mucocutaneous junction and oral mucosa, but adjuvant treatment was pursued in more than 70% of patients afterwards (Chan et al., 2018). Radiotherapy is an effective treatment in human CTCL and its efficacy was demonstrated as a palliative or curative modality in canine CTCL, but longer results were achieved in solitary masses without lymph node involvement (Berlato et al., 2012; Santoro et al., 2017).

Several drugs have been used to treat CTCL with variable results. CCNU, Lyposomal-enriched doxorubicin, dacarbazine, VLC-110 and CHOP-derived protocols showed different responses (Lemari and Eddlestone, 1997; Vail et al., 1997; Williams et al., 2006; Risbon et al., 2006; Morges et al., 2014), however no consensus exists for CTCL treatment. CCNU seems to have more studies than other chemotherapy drugs, complete remissions are achieved in up to 30% and it is widely used in canine CTCL (Laprais and Olivry, 2017), however, free disease interval ranged from 88 to 94 days and it has not showed any beneficial in survival time over multiagent protocols (Risbon et al., 2006; Williams et al., 2006; Laprais and Olivry, 2017; Chan et al., 2018). CHOP protocol resulted effective for achieving first remission in 38% of dogs with multiple cutaneous lesions (Chan et al., 2018), however it is still uncertain in which moment or stage the dog will be more beneficiated from lomustine or CHOP protocol. Rabafocsanide is a new drug with cytotoxic effects on cutaneous lymphoma cells and its use produced a remission rate of 45% in 11 dogs; however, responses were of short time (Morges et al., 2014). Other therapies have been attempted in CTCL including retinoid and linoleic acid that demonstrated beneficial effect in clinical improvement and longer survival time in multiple lesions, interestingly, retinoid showed to be effective independent of chemotherapy concomitant use (Iwamoto et al., 1992; Chan et al., 2018). Linoleic acid in the form of safflower oil was reported to produce remission in 75% of cases of CTCL, however the authors did not discriminate between complete or partial remission and survival times were not clearly defined (Iwamoto et al., 1992). Finally, attempts with the tyrosine kinase inhibitor masitinib was performed in dogs with epitheliotropic lymphoma, but complete remission was achieved in only 25% of patients with a median duration of 85 days. Authors in that study defined that no KIT

receptor was expressed so the response to masitinib is generated by other pathways than via the KIT receptor (Holtermann et al., 2016).

2.6 Prognosis

Prognosis of CTCL is poor (Rook, 2019). Mucocutaneous and mucosal lesions seem to have better prognosis (491 days) when compared to cutaneous lesions (130 days) (Chan et al., 2018). Response to treatment could be related to longer survival time, since some authors demonstrated a statistic difference when complete remissions were achieved (Chan et al., 2018). Many veterinary studies showed poor survival times that ranges from two months and up to 2 years, with a median of survival time of 6 months when treated with lomustine (Risbon et al., 2006; Williams et al., 2006; Fontaine et al., 2009; Duarte et al., 2016; Laprais and Olivry, 2017), and the most recent and largest study of epitheliotropic lymphoma reported an overall median survival time of 264 days (Chan et al., 2018). Few reports exist in Brazil as most of the information came from Europe and Australia. Duarte et al., 2016 described a highly aggressive CL characterized by lack of epiteliotropism in a small population of dogs (n=15) with a poor survival time of 22 days. The poor prognosis in dogs contrasts with the good prognosis of MF in humans, however, two types of human CTCL that have similarities in T-cell phenotype (primary cutaneous aggressive epidermotropic CD8-positive) and T-cell receptor (primary cutaneous γδ T-cell lymphoma) present a poor prognosis and significant shorter survival time when compared to MF (Willemze et al., 2019).

CTCL has been described as a model for human oncology (Moore et al., 1994) and due to poor prognosis in dogs as previously described, the use of new drugs translating from human medicine can impact survival times in dogs with this disease. It is expected that in the near future new strategies for CTCL will be developed and better prognosis achieved.

2.7 Epigenetics

Epigenetic mechanisms are involved in carcinogenesis of multiple malignant tumors in humans, and also in CTCL with treatment implications. The association between epigenetics and cancer took its origin when carcinogenesis could not just be explained only by genetic alterations. In 1942, Waddington first described “epigenetics” as the interaction among genes and their product (Biswas and Rao, 2017), nevertheless, it was until 2009 that the term epigenetics was re defined as the study of changes in genes function without involving a change in the structure of the DNA (Dupont et al., 2009). Alterations in the gene expression with same and intact genome could explain the diverse phenotype identity that characterizes cancer cells (Toh et al., 2017).

Two different but complementary epigenetic mechanisms are related to cancer: DNA methylation and histone modification (Sharma et al., 2010), and more recently evidence suggest that microRNAs expression is dysregulated in cancer through epigenetic changes (Peng and Croce, 2016). In DNA methylation a methyl group is added at the 5’ carbon of the cytosine ring. The resulting 5-methylcytosine is found more commonly within the cytosine-guanosine dinucleotide (CpG). Typically, these CpGs form clusters and locate in relevant regions of the DNA mostly in the promoter or first exon region known as CpG islands (Kulis and Steller, 2010). Basically, CgP islands remain unmethylated in gene active transcription, whereas became methylated in gene inactive transcription or silencing. In germ-line tissue and somatic cells, these CpG islands remain unmethylated and thus, gene expression occurs. In contrast, methylation of CpG islands induces transcriptional silencing to suppress the expression of any harmful sequence that may alter the DNA integrity (Baylin, 2005). The methylation process is regulated by a class of enzymes called DNA methyltransferases (DNMTs), which finally will add the methyl group to the cytosine group (Baylin, 2005). A family of DNMTs are described but only three enzymes may play a role in the methylation process: DNMT1, DNMT3a and DNMT3b (Kulis and Steller, 2010). DNMT1, also called a maintenance enzyme, is involved in restoring the DNA methylation pattern in new formed cells. In the other hand, the family DNMT3

known as *de novo* enzymes, are in charge of the DNA methylation pattern during embryogenesis and germ cell development (Okano et al., 1999).

Currently, two mechanisms explain how the methylation status induces transcription silencing. In one of them, DNA methylation creates a physical barrier, inhibiting the access to promoter binding sites. However, only some transcription factors are explained by this machinery (Kulis and Steller, 2010). The other mechanism associates the histone modification process through methyl-CpG binding domain proteins (MBDs) that are the responsible for recruiting histone deacetylases (HDAC) at specific points in the chromatin. HDAC remove acetyl groups from the histones (deacetylation), compacting the DNA and inhibiting gene expression.

While methylation of the DNA within CpG islands can be associated or not to histone deacetylation to promote transcription silencing, by contrast, histone acetylation is related to active transcription. Histones are crucial proteins in charge of DNA enrolling and because of its role in DNA wrapping, they promote gene activation or silencing. Histones are structurally composed by amino-terminal tails protruding from the nucleosome that are subject to a wide variety of modifications (Fullgrabe et al., 2011). Histone modifications alter the chromosome function through two mechanisms: changing the electrostatic charge that results in a structural change in histones or how they bind to the DNA; and through the recognition of the modifications by bromodomains or chromodomains that recognize acetylated or methylated lysines, respectively (Fullgrabe et al., 2011).

Particularly in CTCL, methylation of the DNA and histone modification seems to play a role in the initial steps of carcinogenesis in T-lymphocytes (Izykowska and Prybylsky, 2011). Several studies have described histone acetylation as one important mechanism related to CTCL carcinogenesis and although cancer development is recognized for having multiple participation of factors, epigenetics has become more recently linked to CL development (Zhao and Tao, 2018). Histone modifications more commonly described include acetylation, methylation, ubiquitination and phosphorylation (Sharma et al., 2010). Among these modifications, histone acetylation is regulated by two antagonist enzymes: histone acetyltransferases (HAT) that add acetyl groups to the histone tails and HDAC that remove acetyl groups from the histone

tails, as discussed previously. In human CTCL, HDAC are overexpressed (Marquard et al., 2008) and they are believed to participate in malignant transformation of lymphocytes through silencing or transcription repression of tumor suppressor genes (Kim et al., 2001). Also, histone acetylation gains more impact in CTCL since inhibitors of HDAC were designed to enhance acetylation of malignant lymphocytes and permit gene transcription of suppressor genes such as p21 (Zain and O´Connor, 2010).

Although HDAC acts potentially on histones inducing deacetylation, its function has emerged in other proteins within cells. Interestingly, retinoids that are metabolites with similar molecular characteristics than vitamin A, present receptors to HDAC (Urvalek and Gudas, 2014). Physiologically, retinoids have a role in cell differentiation, proliferation and in tumor suppressor gene activation (Ren et al., 2005). After retinoic acid – a type of retinoid- bind to its receptor, an interaction occurs with retinoic acid response elements (RARE) that are close to promotor or enhancer regions of retinoic acid regulated genes (Urvalek and Gudas, 2014). In cancer, the use of HDAC inhibitors was confirmed to be effective to reverse the repression of the tumor suppressor gene RARβ2 in renal carcinoma. In addition, it was shown that when used HDAC inhibitor associated with retinoids occurred tumor regression (Wang et al., 2005). This epigenetic signature was also found in mammary carcinomas (Fang et al., 2015) and although it has not been proved in canine tissue, it may represent a new target of HDAC inhibitors in cancer.

Another important mechanism in epigenetics is controlled by microRNAs. MicroRNAs are non-coding RNAs that are dysregulated in cancer by different mechanisms like deletion or amplification of microRNA genes, dysregulated epigenetic changes and abnormal transcriptional control of microRNAs (Peng and Croce, 2016). They are involved in a diverse setting of cellular processes including apoptosis, cell proliferation and differentiation. In normal lymphocyte development, microRNA may play an important role (Auer, 2011), but also they are linked to carcinogenesis through expression of oncogenes or altering the expression of tumor suppressor genes (Kasinski and Slack, 2011). In human lymphoma a different signature of microRNAs can be present in malignant cells than in normal lymphocytes (Auer, 2011). This difference can be “read” by detecting the changes in circulating microRNA expression in blood or plasma from human or canine patients with lymphoma. Although research

has not been reported in CTCL, microRNAs may be promising candidates for understanding better its behavior and for diagnosis or disease monitoring in dogs with this disease.

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CHAPTER 2 - Clinical description and prognostic factors of high grade cutaneous T-cell lymphoma in dogs[1]

Running tittle: High grade cutaneous lymphoma in dogs

Oscar R. Sierra*, Igor S. Senhorello*, Isabela Canavari*, Felipe R. Sueiro†, Giovanni Vargas-Hernandez‡, Roberto Ampuero*, Andrigo Barboza de Nardi*, Mirela Tinucci-Costa*.

*Clinic and Surgery Department, College of Agricultural and Veterinarian Science, São Paulo State University, UNESP, Via de Acesso Prof.Paulo Donato Castellane s/n, 14884-900, Jaboticabal, SP, Brazil.

†VETPAT, Veterinary Pathology Laboratory. R. Cel. Manuel de Morais, 146 - Jardim Brasil, 13073-022 Campinas, SP, Brazil.

‡College of Veterinary and Animal Science, National University of Colombia, Carrera 45 N° 26-85, Bogotá, Colombia.

Corresponding author: Oscar R. Sierra. Clinic and Surgery Department, College of Agricultural and Veterinarian Science, São Paulo State University, UNESP, Via de Acesso Prof.Paulo Donato Castellane s/n, 14884-900, Jaboticabal, SP, Brazil. osirra@hotmail.com

1. Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

2. Sources of funding

This study was supported by grant number 2016/00128-5 from the São Paulo Research Foundation - FAPESP.

[1] This chapter corresponds to the scientific article submitted to the Journal Veterinary Dermatology and it is currently under evaluation for publication

3. Abstract

Background: Cutaneous T-cell lymphoma (CTCL) is a rare dermatologic and systemic condition and limited information exist regarding clinical and histological features of high grade CTCL or advance-stage disease.

Objectives: To describe clinical, histological and prognostic characteristics of a high grade form of CTCL

Methods: A retrospective review of medical records from 2014 to 2018. Clinical information of 21 animals diagnosed with CTCL were collected. Histological slides and CD3, CD79a and Ki67 immunoexpression were evaluated. Median survival time (MST) and time to progression (TTP) were compared regarding epidemiologic characteristics, tumor stage, treatment and response to chemotherapy for statistical and prognostic purposes.

Results: The overall median survival time was 31 days and median time to progression was 10 days. All samples were characterized by large malignant lymphoid cells, deep dermal infiltration and mean proliferative index of 65,9% detected by Ki67 immunoexpression. Thrombocytopenia was associated with shorter TTP ($p=0.03$). Dogs without previous dermatologic disease, thrombocytopenia and positive thoracic involvement seen on radiography at diagnosis presented shorter MST ($p<0.05$). On multivariate analysis, only thoracic involvement seen on radiography was associated with shorter MST (21 days) ($p<0.001$).

Conclusion and clinical significance: Dogs with clinical and histological characteristics of high grade CTCL presented an extremely poor TTP and MST. Thoracic involvement on radiography constituted an independent negative prognostic factor for survival in this population. Whether this form constitutes a late stage of the disease or an aggressive variant of CTCL is still uncertain.

4. Introduction

Cutaneous lymphoma is an infrequent tumor diagnosed in dogs, cats, horses and humans (1, 2, 3, 4). In dogs constitute <1% of all canine cutaneous tumors and represents 3-8% of all lymphoma types (2). The largest study recorded 148 dogs in a 12-year period (5) and others estimated a prevalence of 2-7 cases out of 1000 dogs (6). It is considered as an extranodal lymphoma, due to its origin in the skin and not in lymphoid tissue.

Cutaneous T-cell lymphoma (CTCL) represents most of the cases of cutaneous lymphoma and can be further classify regarding cell size,

localization of lymphocytes in epidermis, expression of CD4, CD8 and CD30 and T-cell receptor phenotype (α/β or γ/δ) (7). A typical CD3+ and CD4+CD8- represents the classic form of human CTCL called Mycosis Fungoides (MF) characterized by its indolent behavior, well-understood clinical stage and good prognosis (8). In MF, T-cells present marked tropism to epidermis and its adnexa (epidermotropism) and constitute a common characteristic of the reported studies of CTCL in dogs (5, 6, 7, 9), while minimum or absent epiteliotropism may be observed in advanced-stages of MF or in Sezary Syndrome, which is defined as the leukemic variant of CTCL (8).

In humans, advanced-stage MF or primary cutaneous peripheral T-cell lymphoma non otherwise specified (PTCL-NOS) represent aggressive forms of CTCL with poorer survival times and more unfavorable prognosis than early-stage of CTCL (10, 11). Previous studies showed that the proliferative marker Ki67 was significantly lower in early stages than advanced stages MF or in primary cutaneous PTCL-NOS (12, 13). In dogs, immunoexpression of Ki67 in canine CTCL was highly variable and association to clinical outcome or prognosis could not been found (6).

In dogs, MF seems to have a more aggressive course than the human counterpart (2). Overall responses to chemotherapy seem to be lower than those obtained for multicentric lymphoma (6, 14, 15) and many veterinary studies showed short survival times, ranging from a couple of months to 2 years (2, 16, 17, 18, 19), with an overall median survival time of 264 days in the most recent and large published study of CTCL in dogs (5). Despite of large documentation of CTCL in Europe and Australia, the disease has been poorly described in America. In Brazil, a study evaluated 15 dogs diagnosed with CTCL treated with CCNU at a high dose (90 mg/m2) with no complete remissions achieved and a poor median survival time of 22 days (19). Interestingly, the majority of dogs in that study (13/15) had no evidence of epitheliotropism. Similarities of prognosis have been seen at our institution and it is the authors´ opinion that a more aggressive form of CTCL exists, in which epiteliotropism is lost and large malignant lymphocytes cells predominate.

In this retrospective study, we aim to describe the aggressive clinical course and prognostic features in a population of dogs diagnosed with CTCL. We show features similar to those found in high grade T-cell lymphomas in the population studied with an extremely poor survival time.

5. Material and Methods

5.1 Ethical statement

This study was approved by the Committee of Experimentation and Use of Animals in research (CEUA) by our institution (protocol number: 010049/17).

5.2. Animals and tissue samples

Cases of confirmed CTCL during 2014 and 2018 were selected for reviewing complete medical record and histology analysis. Dogs were included in the study if a histopathological and immunohistochemistry result was consistent with CTCL and lack of of epiteliotropism was informed. Original haematoxylin & eosin stained slides were reviewed for confirming non-epiteliotropism of malignant lymphocytes and later new slides for CD3 and CD79 immunohistochemical analysis were performed.

5.3 Immunohistochemical staining

Antibodies used in the immunohistochemical stains included rabbit polyclonal anti-CD3 (1:300, Dako), mouse monoclonal anti-CD79a (HM57, 1:100, Dako) and mouse monoclonal Ki67 (MIB-1, 1:100, Dako). Immunostaining was performed as previously described (20, 21). Ki67 evaluation was followed as previous counting method (6). Briefly, an area with the highest proportion of positive cells at 100x was selected. Then, positive cells were counted in a total of 1000 stromal cells at 400x magnification and percentage of Ki67 positive cells was further estimated.

5.4 Clinicopathological variables

Medical records of all selected animals were reviewed by a single investigator, who collected all clinical information to further classify it into dichotomized clinical variables. For each eligible dog, information collected from the anamnesis included sex, breed, age, date of visualization of first cutaneous lesion, presence or absence of previous dermatologic disease, infectious diseases or corticoid treatment before consultation. Information

concerning the cutaneous lesion and disease behavior included tumor size registered in its longest axis (<5cm or ≥5cm), number of nodules (solitary or multiple), presence or absence of ulceration, location (cutaneous, mucocutaneous or mucosal), lymph node status (palpation, cytological or biopsy result) and presence or absence of clinical systemic signs.

Information from paraclinical testing included complete blood count levels at diagnosis (presence or absence of anemia, thrombocytopenia, leucopenia or leukocytosis) and imaging alterations suggestive of metastasis on thoracic X-rays. Data from abdominal ultrasound was recorded, but because infiltration by lymphoma was not confirmed in any organ, we ruled out this variable for any analysis. From treatment and response, chemotherapy drug (CCNU or CHOP-based protocol), response to treatment, date until progression and death were also included. Time to progression (TTP) was considered from the date of treatment initiation until date of progressive disease. Survival time was considered from the date of diagnosis until death due to any cause. Death was confirmed by the owner through phone calls.

All dogs had measurable disease at the diagnosis and cutaneous lesions were registered by its longest axis by caliper measurement. Response to treatment was calculated according to the values described in response evaluation criteria for nodal lymphoma in dogs (22), thus, a complete remission was defined as total disappearance of cutaneous lesions or measurable disease. A partial remission was defined as ≥ 30% decrease in size of measurable disease, a progressive disease was defined as ≥ 20% increase in size of measurable disease or appearance of any new lesion and stable disease was defined when none of the described criteria were met.

5.5 Statistical analysis

For statistical purposes, all variables were dichotomized for comparison purposes. The median survival time (MST) and median TTP was defined for each variable, survival time and TTP curves were estimated and compared among all dichotomized variables through Kaplan-Meier analysis and Log-Rank test. Any factor with $p<0,1$ were included in a multivariate Cox forward logistic regression model. Significance was considered at $p<0.05$ for multivariate analysis. Statistical analyses were performed using Grahpad-Prism 6.0 and SPSS Statistics 25.

6. Results

Twenty-one cases were selected after immunohistochemistry results. CD3+CD79a- was found in 19 samples, whereas CD3+CD79a+ in only two. The median age of the 21 cases was 7 years, ranging from 2 to 14 years. Females seemed to be overrepresented in our study (13/21), when compared to males (8/21). The cross-bred dog was the most common affected (7/21) and pure bred dogs represented more than once were Golden Retriever (2/21), Poodle (2/21), Boxer (2/21). A summary of the clinical information is provided in Table 1.

All dogs had cutaneous nodules and none of them presented exfolliative erythroderma, patches or nodules smaller than 2 cm. Lesions ranging from 2,5 cm until 15 cm. Nodules had a mean size of 6,2 cm. We selected the longest axis as a reliable measure since tumor volume were not registered for all the cases. Two patients (9,5%) presented with a solitary nodule at initial consultation and the remaining 19 reported multiple lesions. Cutaneous lesions were presented mostly on the trunk and abdomen (Figure 1a). Limbs and head/neck region were less encountered. Mucocutaneous form was evident in 7 dogs (33,5%) mostly in mouth, eyelid and genital junctions (Figure 1b). Oral involvement was found in 2 dogs (9,5%) concomitant with cutaneous lesions (Figure 2a). Several dogs presented rounded or ring shaped lesions, commonly thicker at the periphery and depressed in the center (Figure 2b). Because of the shape on the skin, these lesions are also called serpiginous or arciform and have been described in CTCL in dogs (23, 24). Most of the dogs had ulcerated lesions but we could relate this characteristic to worse prognosis. The mean growth time before diagnosis was 99 days, some owners could not define an approximate date of first cutaneous sign. Enlarged loco-regional lymph nodes were increased on palpation in 15 dogs (71%), six dogs (28,5%) performed fine needle aspiration and just two confirmed lymph node involvement after cytology results. We did not associate statistically node involvement and survival time due to lack of precise information regarding node status in terms of cytology or histopathology.

Systemic signs were present in 14 dogs (66,5%). Depression, lethargy, gastrointestinal signs and limb edema were most commonly reported (72%). Dyspnea, lameness, ataxia and ophthalmologic signs were described concomitantly in several cases (52%). In an attempt to establish a relationship between Ehrlichiosis and prognosis of CTCL, we search for patients diagnosed previously with this disease. Seven dogs (33%) had history of Ehrlichiosis but we were not able to associate it with prognosis. Previous dermatopathy was taken as a clinical variable since some studies have associate it with increased risk of developing cutaneous lymphoma. Two dogs had a history of allergic diseases (9,5%) and one of autoimmune

disease (5%). Interestingly, a prolonged median survival time (119 days) was found in dogs previously diagnosed with any dermatopathy.

We also wanted to know if corticoid administration before initiating definite treatment could influence a poorer response to chemotherapy. Eight dogs (38%) reported previous use of corticoid: seven by oral administration and one by topic treatment. History of corticoid administration ranged from few days until four months. Because of low number of cases we were not able to find any association of corticoid administration with treatment response (data not shown).

Histopathology evaluation revealed lymphoid cells located in deep dermis and in some cases, close to adipose tissue in subcutis (Figure 3a). Lymphocytes were compared to erythrocytes to estimate their size; large lymphocytes were defined as twice the size of an erythrocyte, and intermediate lymphocytes as 1,5 the size of an erythrocyte. Despite some cases present with few small lymphocytes (same size than erythrocytes) near the epidermis, all cases had a diffuse infiltration of intermediate to large lymphocytes forming a diffuse pattern in the deep dermis (Figure 3b). Cytoplasm was scarce and nucleoli was evident in most of the samples. Positive CD3+ cells were clearly seen in dermis with minimum tropism to epidermis or its adnexa (Figure 4a). Two cases presented positive immunoexpression to both CD3 and CD79a (Figure 4b). Nuclear immunolabeling of Ki67 in neoplastic cells were clearly identified in all samples (Figure 5). The mean value of Ki67 was 65,9% (37,9-93,9) that corresponded to high grade according previous authors (6). Distribution of Ki67 immunoexpression was variable in all cases, some samples presented positive cells close to epidermis while others in the subcutaneous tissue.

Anemia (hematocrit <37% or hemoglobin <12/dL) was commonly reported in blood exams at diagnosis. Ten dogs had anemia (48%) classified mostly as normocytic normochromic. Seven dogs had hematocrits above 30% and three below 30%; the lowest hematocrit registered was 21,2%. Thrombocytopenia (<180000 cells/µL) was found in 8 dogs (38%), while twelve (57%) presented number of platelets within reference range. Ehrlichiosis was suspected in cases of thrombocytopenia, but it was only confirmed in one case through antibody detection using a commercial test. This dog presented the lowest number of platelets (65000 cells/µL) and received doxycycline concomitant with prednisone, no chemotherapy was given. Median TTP (7 days) and MST (22 days) of dogs with thrombocytopenia were statistically different from dogs with normal number of platelets (21 days, 48 days, respectively) ($p<0.05$). Five dogs (20%) presented leucopenia and four (16%) leukocytosis due to elevated neutrophil number. Biochemical results were within normal reference value for canine in almost all the population studied. Exceptionally, one dog had increased creatinine level (3,2 mg/dL) and died 6 days after diagnosis (this

dog was excluded for all survival analyses) and 6 dogs (28,5%) presented elevated ALT levels (>80 U/L).

Thoracic radiography was available for evaluation in 19 animals. Five dogs (24%) presented abnormal findings that included increased tracheobronchial and sternal lymph nodes, pericardial and pleural effusion. Infiltration by lymphoma was the most likely diagnosis in the patients with increased intra-thoracic lymph nodes (n=3) due to absence of clinical signs suggestive of other respiratory disease and lymphoma was demonstrated by cytology of the pleural and pericardial effusions (n=2). Dogs that show thoracic involvement seen on radiographies presented a statistically poorer MST (21 days) than dogs without involvement as will be explained in next section. Abdominal ultrasound was performed in 17 dogs. Ten of them (48%) presented abnormal findings that included enlarged iliac lymph nodes, hepatosplenomegaly, nodules in spleen and altered echotexture of liver. Infiltration by lymphoma was not confirmed in any organ.

Chemotherapy using lomustine (60-80 mg/m2 every three weeks) with or without prednisone was performed in 15 dogs (71%), while the CHOP protocol (vincristine, cyclophosphamide, doxorubicin and prednisone) was attempted in one patient that lived 57 days. Prednisone as a sole treatment was performed in one patient that lived 24 days. Four dogs received no treatment (19%) and survival time was not different for treated dogs (41 days) versus non-treated dogs (24 days) ($p>0.05$). Treatment response was discouraging, since 10 dogs (48%) presented progressive disease and just seven patients (33%) any biologic response (CR, PR or SD). Four dogs did not report treatment response because this information was not available or died before any objective response could be achieved. Three dogs (16%) received a second treatment option that included CHOP protocol (n=2) and lomustine (n=1).

6.1 Prognostic factors

The MST for 21 dogs was 31 days and the median TTP was 10 days. One dog was censored because died of renal failure 6 days after diagnosis. Just three dogs (14%) lived over 100 days (114, 119 and 155 days). Two of them had multiples nodules affecting cutaneous and mucocutaneous regions, but nodules were in all cases smaller than 5 cm.

Complete blood count was available for 20 animals. For all variables evaluated, only thrombocytopenia showed a statistical association with TTP and survival time. Six dogs presented with thrombocytopenia at diagnosis and showed shorter median TTP (7 days) than dogs without thrombocytopenia (21 days) ($p=0.03$) (Figure 6). The MST for thrombocytopenic and non-thrombocytopenic populations were 22 days and

48 days, respectively (p=0.02) (Figure 7). Table 2 presents the defined prognostic values for survival time.

Thoracic radiography was evaluated in 19 animals. Thoracic involvement of lymphoma was positive in five dogs (24%). Dogs with positive thoracic involvement lived shorter (21 days) than dogs with negative thoracic involvement (52 days) (p<0.0001) (Figure 8). Animals with thoracic involvement also presented shorter median TTP (8 days), when compared to animals with normal X-rays (17 days), but this difference was close to be statistically significant (p=0.051). Table 3 presents the prognostic factors for TTP and its value of significance.

Interestingly, three dogs (12%) presented a history of dermatologic disease that included allergic dermatitis (n=2) and autoimmune disease (n=1). These three dogs presented a MST of 119 days that was different from dogs with no history of dermatologic disease (24 days, p=0.04) (Figure 9).

6.2 Multivariate analysis

Multivariate analysis was performed for defining prognostic factors for TTP and survival time. On univariate analysis, presence of thrombocytopenia was associated to shorter TTP (p=0.03), but on multivariate analysis none of the variables retained statistical significance (p>0.05) (Table 3). As well as for TTP, presence of thrombocytopenia was potentially associated with survival time on univariate analysis (p=0.02), but this was not confirmed on multivariate analysis. Presence of clinical signs (p=0.06), nodule size (p=0.056), dermatologic disease (p=0.04) and thoracic involvement (p<0.0001) were also analyzed on the cox regression model for survival time. After multivariate analysis, only thoracic involvement on radiography (p<0.0001) resulted to be significant. Based on univariate and multivariate results, thoracic involvement seen on radiography constituted an independent prognostic factor for poor survival (Table 2).

7. Discussion

In this study we describe the clinical presentation, rapid evolution and poor prognosis of 21 cases diagnosed with CTCL that had an aggressive clinical behavior and poor response to chemotherapy. The 21 dogs in this study had a median age lower (7 years) than reported in other studies. Most of the authors have described an older population (11 years) of dogs

committed with CTCL (5, 6, 7, 9, 25), however, other authors reported a younger age (8 years) in patients with non-epitheliotropic cutaneous lymphoma (26). Most of the affected dogs in our study were females as described in recent studies (5, 25) and some others have not found a female predilection (6, 19). The cros-bred dog was overrepresented in this study as it seems to be true for other authors (5, 7, 19, 25). Because of low number of dogs in this study, breed predisposition could not be established.

The clinical presentation of our population was strictly nodular and mostly cutaneous. It has been described that CTCL has a variable clinical manifestation and initial forms like exfolliative erythroderma or patch/plaque forms may progress to tumor lesions (2). The fact that our population presented with tumor stage lesions at first consultation could be the result of a lack of effective veterinary assistance at initial stages (owners did not bring the dog to a veterinary consultation or wrong diagnosis made by a veterinarian) or because a more aggressive form of disease was established rapidly in these dogs. The latter explanation is supported by the short time of first onset of disease until diagnosis reported in this study (mean of 99 days). Authors (25) reported a period of time of 64 days in a population of dogs that lived 99 days with CTCL. Other authors have reported prolonged times of development of cutaneous lesions, ranging from 5,4 to 7 months (6, 7, 9). We found that many owners reported to have noticed a nodule as first cutaneous lesion and brought directly to the oncology service of this institution. However, one dog reported to have had spontaneous remission of some cutaneous nodules similar in shape and size 11 months before CTCL was diagnosed. Since dates of onset of cutaneous lesions were approximately deduced by owners and not all animals had this information, a clear association between rapid growth of cutaneous lesions and a more aggressive behavior cannot be truly established in this study.

Multiple lesions were more commonly found (19/21) than solitary nodules, however some owners reported an initial unique nodule with sudden onset of multiple lesions. Multiple cutaneous lesions constitute the most frequent form of CTCL described in all veterinary literature (5, 6, 9, 26). Animals with solitary lesions may have a better prognosis than dogs with multiple lesions (5), but this fact was not confirmed in our study because just two dogs presented with a solitary mass. We wanted to establish an association between nodule size and prognosis, but large tumors (≥ 5 cm) presented similar median TTP and MST in the population studied. In humans, tumor burden is associated with staging of CTCL at initial stages; less than 10% of skin covered in red patches or plaques means a lower stage than humans with more than 10% of skin affected (27). Veterinary medicine lacks of a staging system for dogs with CTCL, but based on recent evidence (5), solitary lesions may have a lower stage than multiple lesions.

Location of CTCL was more commonly found on the skin (cutaneous form), when compared to mucocutaneous or mucosal location. Almost all dogs presented with nodules on the trunk and abdomen, and some of them had simultaneous nodules on mucocutaneous junctions and oral mucosa. This distribution was found in several other studies, and location generally overlapped among the three main forms of CTCL (5, 6, 7, 17, 25). The characteristic arciform or serpiginous lesion found in many dogs with CTCL has already been described in dogs and humans (23, 24); in the latter has been described in the patch/plaque stage of the disease (28). As it seems to be a common characteristic in CTCL in our region, CTCL should be suspected in dogs with typical arciform or serpiginous lesion until proven otherwise.

Prognosis based on lymph node involvement at diagnosis is still controversial (5, 29). We did not find an association between lymph node metastasis and worse prognosis, however, lymph node status was not evaluated in all dogs of this study. One other study showed a significant survival advantage in dogs with no evidence of lymph node metastasis (29), and recently, another study found no association between lymph node status and survival time (5). Based on systemic signs, lymphoma can be substaged A (absence of systemic signs) or B (presence of systemic signs). Substage has been already defined as a relevant prognostic factor for dogs with multicentric lymphoma, as it means a more advance stage; dogs substaged B presented historically worse prognosis than dogs substaged A (30, 31). However, based on our results, substage B did not impact the survival time of dogs with CTCL and may not be included as a prognostic factor.

Some previous studies have documented dogs with chronic dermatitis before diagnosis of CTCL (23, 32, 33) as it seems to be a link of cutaneous lymphoma and dermatitis in humans (34, 35). Some authors (36) demonstrated an increased risk of developing CTCL in dogs with atopic dermatitis 12 times higher than normal dogs. However, other authors (6) reviewed 30 cases of CTCL and none of them had a previous history of chronic dermatitis. In this study, three dogs presented a previous dermatopathy and had an extended survival time. Two of them had allergic dermatitis and the another one a diagnosis of pemphigus on the nasal planum. These three dogs presented complete response at initial chemotherapy and had a less aggressive course of the disease. No studies have compared survival time in dogs with previous dermatologic diseases and although a possible relationship may exist between dermatologic disease and CTCL, further studies need to prove its association.

Most of the samples (84%) were characterized by a deep infiltration of malignant cells in dermis and in the subcutaneous tissue. All cases had a strong CD3+ immunoexpression. The majority of veterinary literature in cutaneous lymphoma has been described the epitheliotropic CTCL (2, 5, 6,

7, 9, 16, 17, 25), while little information is currently available for non-epitheliotropic CTCL (19, 26). In this study, cells were arranged in a diffuse pattern in the dermis, cell size was predominantly large and few small lymphoid cells were observed close to epidermis. Malignant lymphocytes expressed a high mean value of Ki67 of 65,9%. In accordance to previous Ki67 values in CTCL (6), none of the dogs in this study presented a low proliferative activity (<30%), six, moderate (30-61%) and 15, high proliferative activity (>61%). These results indicate that our population of dogs had a high grade CTCL and this could explain its poorer prognosis. Similar results had already been described in Brazil (19).

In humans, a more detailed immunohistochemical evaluation using CD4, CD8, CD20, CD30 and CD279, and a molecular distinction of the T-cell receptor (αβ or γδ) is performed to categorize a specific type of CTCL (8). In veterinary medicine, immunohistochemical expression of CD4 and CD8 is performed from frozen material and although T-cell receptor profile was already investigated (7), no current techniques are available to identify it. Unfortunately, in this study, frozen samples were not available for CD4 and CD8 immunophenotyping and no investigation of T-cell receptor was further performed. Based on the differences seen in histological infiltration, cell size, high level of Ki67 immunoexpression and poor prognosis, we believe that our population of dogs suffered from a more aggressive form of CTCL that may have a distinct molecular profile. Currently, a prospective study is ongoing to try to elucidate these questions.

In this study, anemia was found in 48% of dogs, however it was mild in most of the cases. Anemia was correlated to poor prognosis in multricentric lymphoma (37, 38), but studies associating anemia in CTCL are lacking in the literature. In our study, anemia was not considered a negative prognostic factor, but it seemed to be a common laboratorial finding in cases of CTCL. Anemia related to cancer is typically normocytic normochromic (38) as described in our population of dogs, therefore, a systemic involvement due to the tumor was highly suspected.

Dogs with lymphoid neoplasia has an increased risk to present thrombocytopenia at the time of diagnosis (39). In this study, 38% of dogs had thrombocytopenia at diagnosis and these dogs presented a shorter TTP and survival time. Similar results were found in canine multicentric lymphoma and humans with high grade T-cell lymphomas, where thrombocytopenia impacted the time of remission in dogs and constituted a negative prognostic factor in humans (40, 41, 42). Although the reason for thrombocytopenia correlating with progression disease and survival time is uncertain, previous studies showed that immune mediated disease or bone marrow infiltration may play a role (41, 43).

In this population of dogs, we found a difference in survival time in dogs having abnormal thoracic radiographies. Thoracic involvement seen

on radiographies was defined as an important negative prognostic factor on univariate and multivariate analysis. Although histologically confirmation of lymphoma was not obtained in the clinical records of patients with intrathoracic lymphadenomegaly, a lymphoid infiltration was assumed to be the only cause of enlarged lymph nodes. Effusions were confirmed as having malignant lymphocytes. Two previous studies reported minimal changes in thoracic and abdominal imaging in dogs with CTCL (16, 17), and no correlation was found with prognosis. As defined previously in multicentric lymphoma (44), the presence of cranial-mediastinal enlarged lymph nodes has a negative correlation to treatment response and survival time. In humans, the presence of extracutaneous disease, defined as distant lymph node or visceral involvement, constitutes an important clinical predictive factor for survival in CTCL (45). Our findings suggest that intra-thoracic involvement could be the result of a more advanced stage in this population of dogs, therefore, a poor response to treatment and survival time may be expected.

Chemotherapy and oral corticoid were the only attempted treatment. Surgery was not performed in any animal possibly because the presence of multiple masses at first consultation. Although some authors have reported a considerable long survival time after surgery of solitary lesions for over 500 days (5), we observed rapid development of new lesions in the two dogs with solitary lesions at 7 and 28 days after diagnosis. Radiotherapy, although available, is not a conventional treatment for dogs in our region due to its expense and equipment localization. Lomustine has been widely used as first treatment option or as a rescue protocol in dogs with CTCL (16, 17), and reported CR and PR ranged from 0-30% and 46-61%, respectively (16, 17, 19). Most of our dogs reported progressive disease and treatment response to lomustine was poorer than previously discussed (14, 16, 17). Based on our results, treated and non-treated animals had no difference in survival time. This means that in our study, dogs presented a rapid progression of the disease even on chemotherapy. Disease progression and drug resistance are fatal consequences in dogs with multicentric lymphoma and in advanced states of CTCL in humans and dogs (2, 45, 46). The cause of death in our population of dogs was associated with systemic involvement of lymphoma, detrimental clinical condition and loss of quality of life.

Our study differs to many others since median survival time reached in our dogs (31 days) can be only compared to one study made in Brazil in 2012 that reported a MST of 22 days (19). The mean TTP of 10 days is the lowest time ever reported (16, 17, 47, 48). A previous review in 2009 (2) described a MST in Europe and North America from 2 months (without any treatment) and up to 2 years (with treatment) and more recently, authors in Australia reported a MST of almost 9 months (264 days) (5).

We believe that dogs in this study lived shorter because they presented a high grade of CTCL at diagnosis or were diagnosed in an advanced stage of MF. Large cell transformation can happen in late stages and can be observed histologically as large lymphocytes invading epidermis, dermis and subcutis (49). Also, minimum or even loss of epiteliotropism is seen histologically during advance-stage MF or sezary syndrome (8). This phenomenon could explain the visualization of malignant lymphocytes at different skin levels in this study.

Our study has some limitations due to its retrospective nature. One important limitation is the lack of frozen material to identify more specifically T-cell immunophenotype CD4, CD8 and T-cell receptor profile. Another limitation was incomplete information regarding staging methods obtained from medical records. Since disease progressed in some cases so rapidly, TTP was not completed in all animals and deaths could be biased by owner decision of euthanasia, thus limiting accuracy of survival time.

8. Conclusion

In conclusion and based on our results, we report a different and more aggressive form of CTCL characterized by large lymphoid malignant cells, absence of epitheliotropism and high immunoexpression of Ki67 that present a poor treatment response and extremely short survival times. Prognostic factors like thrombocytopenia, previous dermatologic disease, and thoracic involvement detected by radiography had significant effect on survival time, but thoracic involvement constitutes an independent negative prognostic factor that influenced survival time. New studies are being conducted to establish a precise immunophenotype of this type of CTCL in our region.

9. References

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10. Tables and figures

Table 1. Descriptive clinical information of 21 dogs with cutaneous T-cell lymphoma

Clinical variable		**N**	**%**	**Mean (range)**
Sex	Male	8	38	
	Female	13	62	
Breed	Cros-bred dog	7	33,5	
	Golden Retriever	2	9,5	
	Poodle	2	9,5	
	Boxer	2	9,5	
	Other pure bred dogs	8	38	
Age				7 years (2-14)
Tumor size (longest axis)				6,2 cm (2,5-15)
Number of nodules				8,2 (1-19)
Body region	Head and neck	9	43	
	Extremities	10	48	
	Trunk and abdomen	20	95	
	Mucocutaneous junction	7	33,5	
	Oral mucosa	2	9,5	
Time from first cutaneous lesion				99 days (15-365)
Ulceration	Absent	10	48%	
	Present	11	52%	
Systemic signs	Lethargy, depression	5	24%	
	Hyporexia, vomit, diarrhea	5	24%	
	Edema in limbs	5	24%	
	Others	11	52%	
Lymph node (on palpation)	Normal	5	24%	
	Enlarged	15	71%	

Previous diseases	Ehrlichiosis	7	33%
	Dermatologic disease	3	14%
Previous corticoid administration	No	10	48%
	Yes	8	38%
Anemia	No	10	48%
	Yes	10	48%
Thrombocytopenia	No	12	57%
	Yes	8	38%
Leucocytes	Normal	11	52%
	Low	5	24%
	High	4	19%
Thoracic involvement on X-rays	Negative	14	67%
	Positive	5	24%
Abdominal ultrasound	Normal	7	33%
	Abnormal	10	48%
Treatment	Lomustine	15	71%
	CHOP	1	5%
	Corticoid	1	5%
	None	4	19%
Response	CR	1	5%
	PR	3	14%
	SD	3	14%
	PD	10	48%

Table 2. Statistically significant association of the most representative clinical variables with survival time

			Univariate analysis		Multivariate analysis	
Variable		**N**	**MST**	***P* value**	**HR (95%CI)**	***P* value**
Tumor size (longest axis)	< 5 cm	6	70	0.05	3,3 (0,7-15,5)	0.1
	≥ 5 cm	14	26			
Clinical signs	Absence	7	59	0.06	4,3 (0,9-20,7)	0.06
	Presence	13	24			
Dermatologic disease	Absence	14	24	**0.04**	0,15 (0,2-1,2)	0.08
	Presence	3	119			
Thoracic involvement on X-Rays	Negative	13	52	**<0.0001**	24,1 (2,6-217,6)	**0.005**
	Positive	5	21			
Thrombocytopenia	Absent	11	48	**0.02**	1,9 (0,6-5,9)	0.2
	Present	8	22			

MST: Median survival time
HR: Hazard ratio (95% confidence intervals for hazard ratio)

Table 3. Statistically significant association of clinical variables with median time to progression

			Univariate analysis		Multivariate analysis	
Variable		**N**	**TTP**	***P* value**	**HR (95%CI)**	***P* value**
Thrombocytopenia	Absent	9	21	**0.03**	3,2 (0,8-12,8)	0,09
	Present	6	7			
Thoracic involvement on X-Rays	Negative	10	17	0.05	3,1 (0,7-12,5)	0,1
	Positive	4	8			
Tumor size (longest axis)	<5cm	5	28	0.07	2,7 (0,7-10,7)	0,1
	≥ 5 cm	10	7			

TTP: Median time to progression
HR: Hazard ratio (95% confidence intervals for hazard ratio)

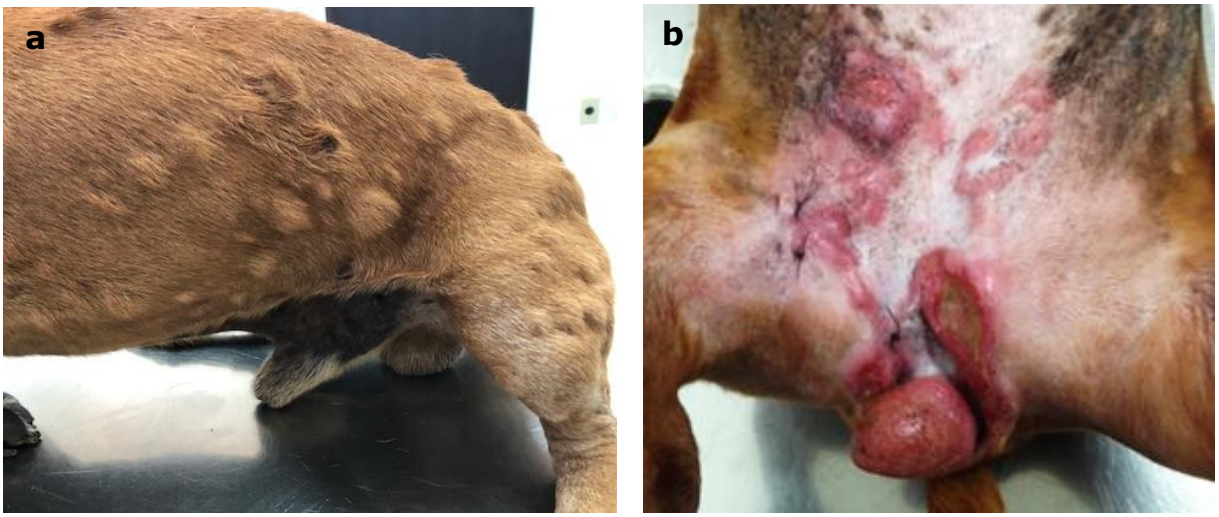

Figure 1. Cutaneous and muco-cutaneous lesions in dogs with cutaneous T-cell lymphoma. (a) Multiple nodules of different sizes are evident on the lateral aspect of this dog, the nodules were also distributed on the other half part of the body. (b) Serpiginous and erythematous lesions in the ventral abdomen of this female dog. A large nodule is involving the muco-cutaneous region in the vulva.

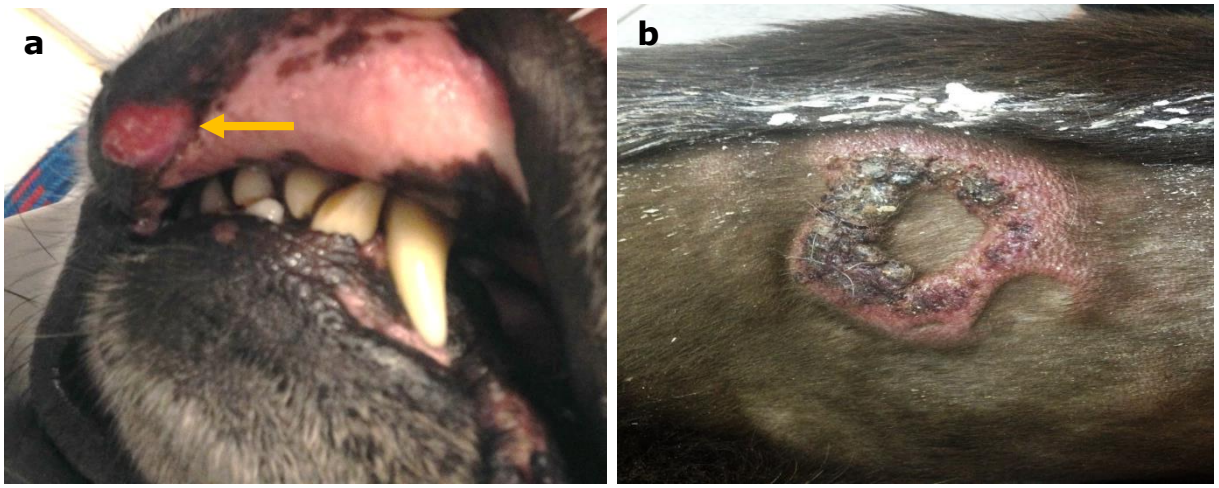

Figure 2. Cutaneous lesions of dogs diagnosed with cutaneous T-cell lymphoma. (a) A single nodule is observed on the cranial aspect of the superior lip in this dog (arrow). (b) Cutaneous arciform lesion observed as typical characteristic in dogs with cutaneous T-cell lymphoma

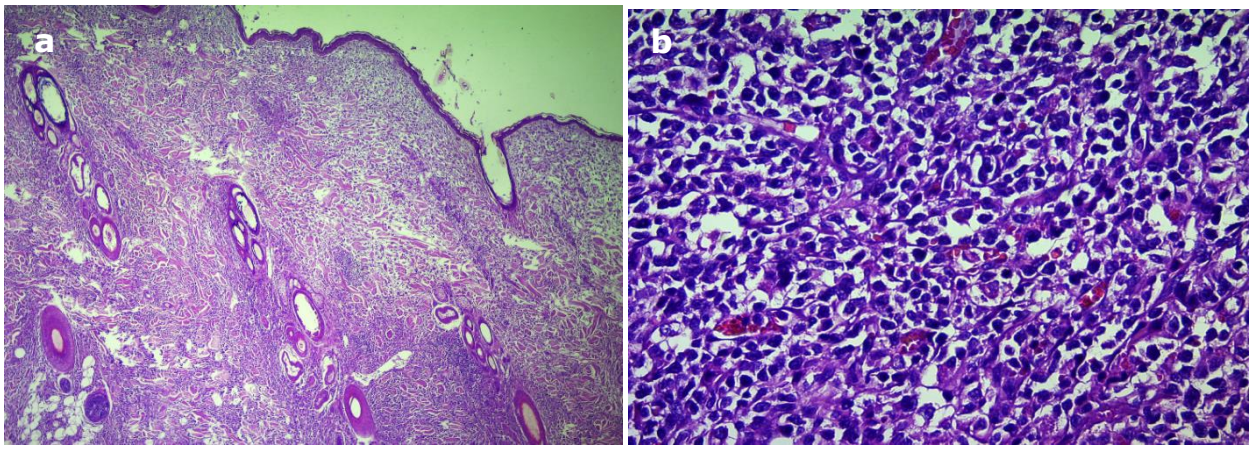

Figure 3. Photomicrography of a skin section from a dog with cutaneous T-cell lymphoma. (a) Diffuse infiltration of malignant lymphocytes expands from close to epidermis until dermis and subcutis (haematoxylin and eosin x5). (b) Intermediate to large rounded lymphocytes are evident in dermis compounding the diffuse pattern (haematoxylin and eosin, x40).

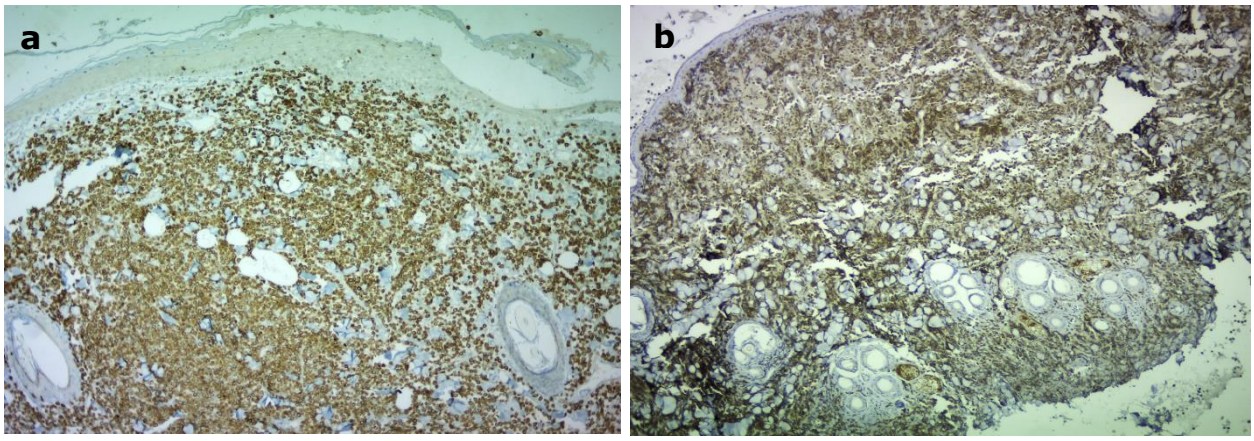

Figure 4. Photomicrography of a skin section from a dog with cutaneous T-cell lymphoma CD3+CD79a+. (a) Positive immunoreactivity for CD3 (CD3, x10). (b) Positive immunoreactivity for CD79a (CD79a, x10).

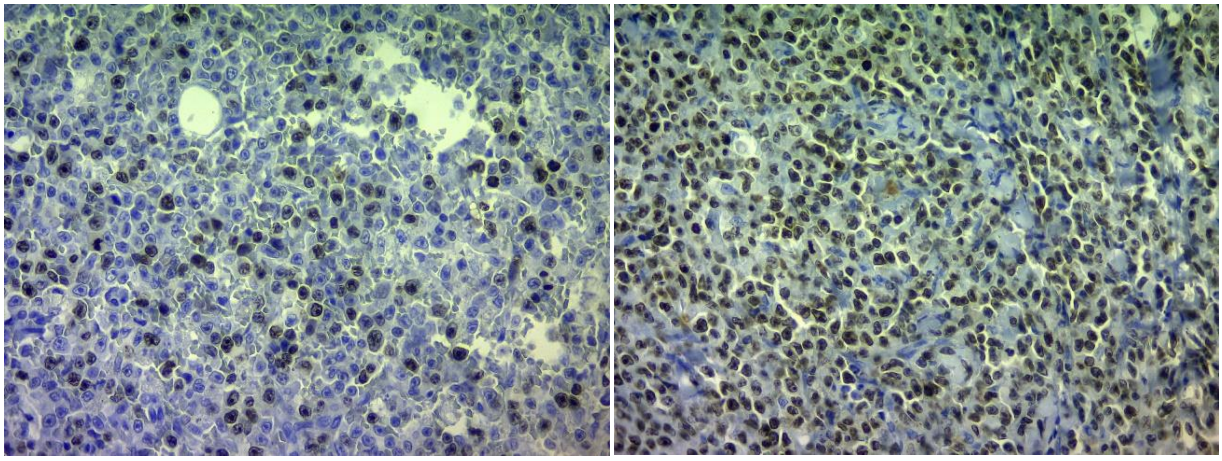

Figure 5. Photomicrography of a skin section immunostained with Ki67 from two different dogs with cutaneous T-cell lymphoma. Sheets of neoplastic cells expressed variedly positive reaction to Ki67; in this case (left) Ki67 had a value of 48%, (x40). Most of neoplastic cells are positive to Ki67 in this case (right); Ki67 value of 79%, (x40)

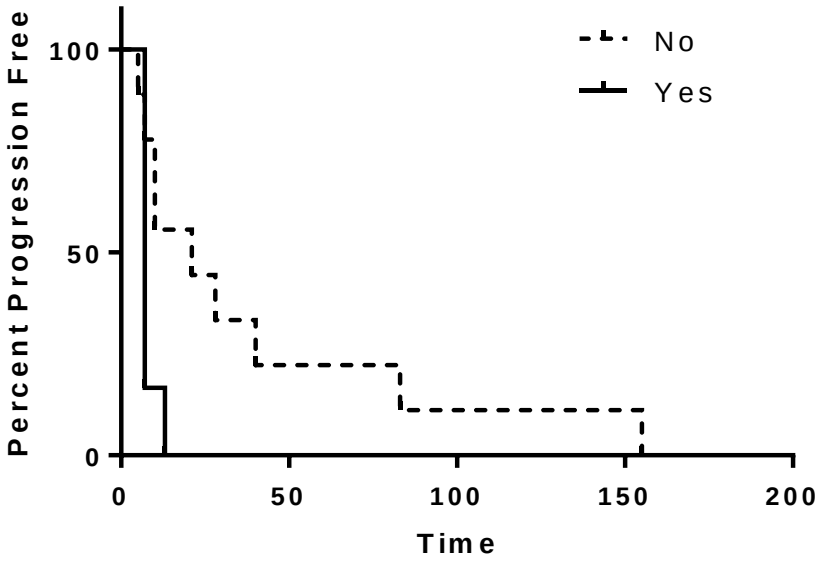

Figure 6. Time to progression curve in dogs with cutaneous T-cell lymphoma based on thrombocytopenia. Dogs with thrombocytopenia (n=6) had shorter time to progression time (7 days) than dogs without thrombocytopenia (n=9) (21 days). P=0.03

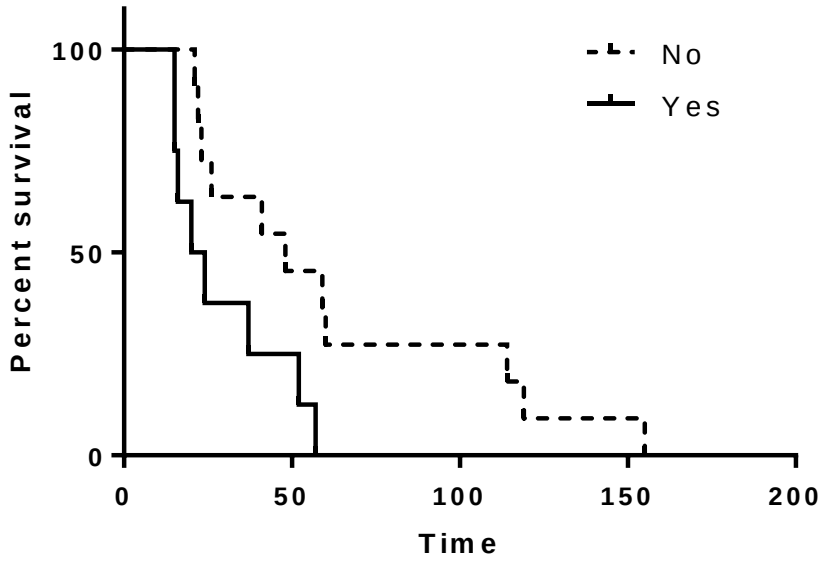

Figure 7. Survival curve of dogs diagnosed with cutaneous T-cell lymphoma based on thrombocytopenia. Dogs with thrombocytopenia (n=8) had shorter survival time (22 days) than dogs without thrombocytopenia (n=11) (48 days). P=0.02

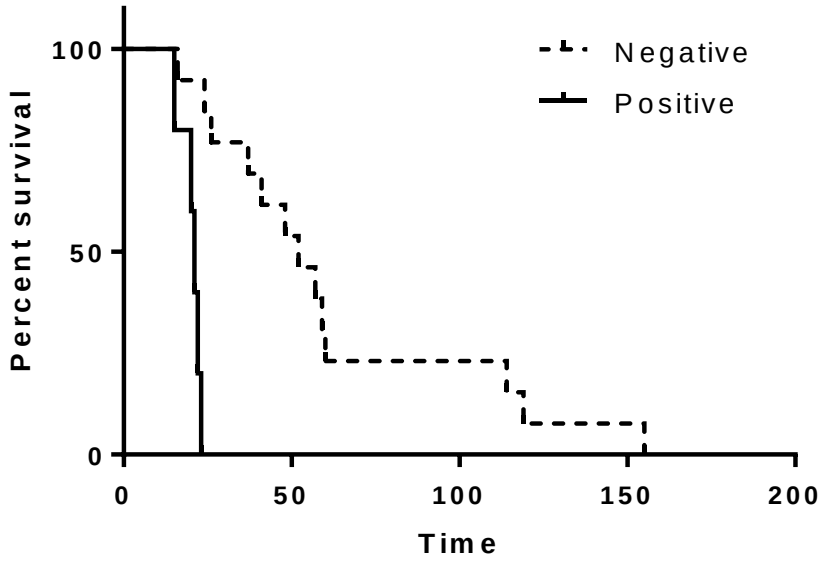

Figure 8. Survival curve of dogs diagnosed with cutaneous T-cell lymphoma according to thoracic involvement seen on radiography. Dogs with negative thoracic involvement (n=5) lived longer (52 days) than dogs with positive thoracic involvement seen on X-rays (n=13) (21 days). P<0.0001

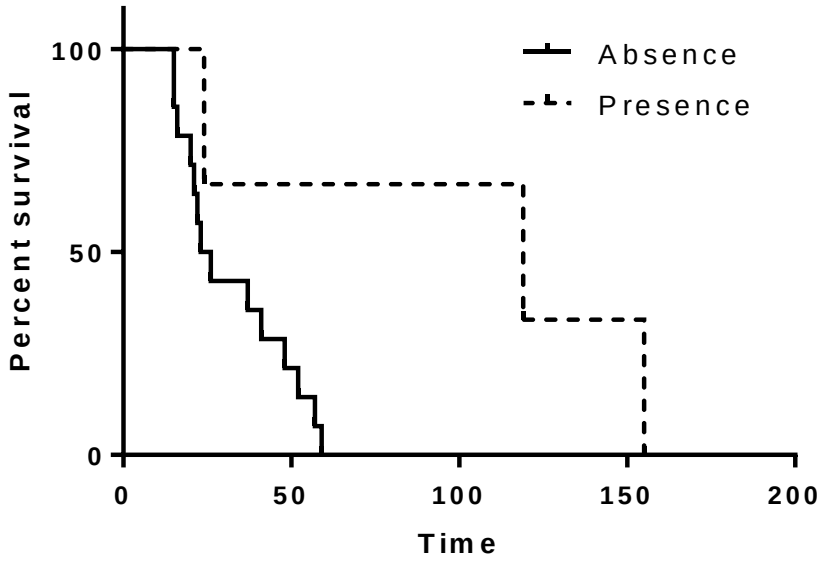

Figure 9. Survival curve of dogs diagnosed with cutaneous T-cell lymphoma according to previous dermatologic disease. Dogs with history of presence of dermatologic disease (n=3) lived longer (119 days) than dogs with absence of dermatologic disease (n=14) (24 days). *P*=0.04

Chapter 3 - Expression profile of acetylated histones 3 and 4 and histone deacetylases 1, 2 and 6 and their association in cutaneous T-cell lymphoma in dogs

1. Abstract

Cutaneous T-cell lymphoma (CTCL) is an uncommon and aggressive type of lymphoma in dogs. Histone modification is one of the main mechanism of epigenetics and association between histone acetylation and treatment in cases of human CTCL have been evolved in recent years, however, no information exists of histone acetylation in dogs with CTCL. This study aimed to characterize the protein level and expression profile of acetylated histones H3 (H3Ac) and H4 (H4Ac) and the histone deacetylate enzymes HDAC1, HDAC2 and HDAC6 in dogs diagnosed with CTCL. Retrospectively, a review of medical records from 2014 to 2018 was performed. Available histological slides, frozen tissue and clinical information were collected from dogs with CTCL. Western blot (WB) and immunohistochemical staining for H3Ac and H4Ac and HDAC1, HDAC2 and HDAC6 was performed and compared among groups CTCL, allergic dermatitis, normal lymph node and normal canine skin. Principal component and exploratory factor analyses were performed to interrogate associations among antibodies. Six and 25 samples of CTCL were available for WB and immunohistochemistry, respectively. Samples of CTCL presented a higher level of H4Ac than other markers ($p<0.05$) and all proteins levels were higher in CTCL than in normal skin cells. By immunohistochemistry, H4Ac and HDAC2 were abundantly expressed in samples of CTCL ($p<0.01$). Malignant lymphocytes presented lower H3Ac levels when compared to normal lymphoid tissue (0<0.01) and dogs with high immunoexpression of H3Ac had longer median survival time (52 days) than dogs with low H3Ac (23 days, $p=0.035$). On multivariate analysis, we estimated an aberrant histone modification pattern (association of H3Ac, HDAC2 and H4Ac) that classified the population in two groups with different survival times (13 days Vs 48 days, $p=0.06$). Canine CTCL shows low protein levels of H3Ac, while high of H4Ac, indicating an aberrant acetylation pattern. Medium levels of HDAC1, HDAC2 and HDAC6 differ

significantly from those found in normal skin cells. The association of H3Ac, HDAC2 and H4Ac differentiate a population of dogs with worse survival. Our results need to be confirmed in further studies in order to translate the use of HDAC inhibitors in dogs with CTCL.

2. Introduction

Cutaneous T-cell lymphoma (CTCL) constitute a type of extra-nodal lymphoma originated from malignant T-lymphocytes that exhibit tropism to the skin (Fontaine et al., 2009). In dogs, although rare, represents 3-7% of all lymphomas (Fournel-Fleury et al., 2002) and it is characterized by a highly variable clinical manifestation that include a generalized erythema with scaling and pruritus –known as exfolliative erythroderma- or patches, plaques and solitary or multiple nodules distributed throughout the superficial area of the body or even close to mucocutaneous junctions and in mucosa (Fontaine et al., 2009; Fontaine et al., 2010; Moore and Olivry, 1994). In contrast to the human counterpart, canine CTCL presents a more aggressive course and is considered to have a poor prognosis (Chan et al., 2018).

Two molecular features have been recognized as the main differences between canine and human CTCL: immunophenotype of T-cells and type of T-cell receptor (TCR) (Moore et al., 2009). Immunophenotype of T-cells in dogs with CTCL is characterized by a lack of CD4 expression (CD4-) and a positive expression of CD8 (CD8+). Eventually, a double CD4-CD8- is also described (Moore et al., 2009). In humans, T-cells are commonly CD4+CD8- and only 10-15% of cases are CD4-CD8+ (Willemze et al., 2019). Furthermore, TCR γδ in dogs with CTCL is recognized in 60% of cases of CTCL, while TCR αβ constitutes the 40% remaining (Moore et al., 2009). In human the most described TCR phenotype is TCR αβ, while TCR γδ characterizes a more atypical and less common form of CTCL (Willemze et al., 2019). Although these differences have been highlighted in several studies, it is still unknown whether they can explain the more aggressive behavior of CTCL in dogs. In the most recent and largest study of canine CTCL dogs lived a median of 8 months being treated with

different treatment modalities (Chan et al., 2018), and other authors have described a median of 6 months using only chemotherapy (Risbon et al., 2006; Williams et al., 2006; Laprais and Olivry, 2017).

Treatment of human CTCL is based on multiple modalities, early-stage lesions are treated with topical agents that include corticosteroids, nitrogen mustard, retinoids/rexinoids, Toll-like receptor agonist (imiquimod) and local modalities like phototherapy with psolaren plus ultraviolet A (PUVA), narrowband ultraviolet B (NB-UVB) and total electron beam irradiation (Hermann et al., 1995; Querfeld et al., 2005; Li et al., 2012). Emerging therapies were further developed in advanced-stage CTCL and immunotherapy including monoclonal antibodies and histone deacetylase inhibitors were added to conventional chemotherapy to provide longer responses and survival times in human CTCL (Kim et al., 2007; Olsen et al., 2007; Querfeld et al., 2009; Li et al., 2012). In veterinary, local therapies are not currently suggested because of limited proved efficacy and generalized disease at diagnosis (De Lorimier, 2006). Patients with solitary lesions performed surgically removal and advanced-stage disease are treated based on chemotherapy, retinoid and/or corticosteroids (Fontaine et al., 2009; Chan et al., 2018). Newer therapies are needed for advanced-stage CTCL in dogs.

Histone modifications are considered a crucial epigenetic mechanism that lead to alter gene transcription in cancer cells with no modification of the DNA sequence (Sharma et al., 2010). These modifications occur in only few residues of the histones tails and include acetylation, methylation, phosphorylation and ubiquitination (Fullgrabe et al., 2011). Among these modifications and in a global way, histone acetylation plays an important role in gene transcription, whereas histone deacetylation involves transcriptional repression or silencing (Khan and La Thangue, 2008). These processes are regulated by two enzymes, one that adds acetyl groups to histones called histone acetyl transferase (HAT) and the another one that removes them called histone deacetylase (HDAC). In human CTCL, emerging drugs like HDAC inhibitors are being used since 2008 when the first HDAC inhibitor –Vorinostat- was released as a therapy for advanced-stage CTCL (Marks and Breslow, 2007). Many different HDAC inhibitors have been used in clinical trials in CTCL (Khan and La Thangue, 2012), and although an exact mechanism of action is still unclear (Sardiu et al., 2014; Ding et al.,

2016), it is believed that benefit of these drugs are product of multiple roles of HDAC inhibitors in malignant cells including apoptosis presumably by reactivating silenced genes (Khan and La Thangue, 2012). By inhibiting deacetylation, cancer cells reverse transcriptional silencing of tumor suppressor genes like p53 and pRb resulting in inhibition of tumor growth (Khan and La Thangue, 2012; Lane and Chabner, 2009).

HDACs are overexpressed in several human malignancies (Chen et al., 2015), and some authors have demonstrated their high expression in multicentric lymphoma and CTCL (Marquard et al., 2008; Marqueard et al., 2009). CTCL presents a higher therapeutic response to HDAC inhibitors in advanced-stage CTCL, showing overall responses rates up to 46% (Lopez et al., 2018). In veterinary, dogs with CTCL finally died because of chemotherapy resistance and disease progression (Fontaine et al., 2009), and no other therapies are attempted in advanced-stage.

Due to dysregulation of H3Ac, H4Ac and HDAC1, HDAC2 and HDAC6 in human CTCL (Marquard et al., 2008) and the urgent search for new therapies in dogs with the same disease, we wanted to evaluate the protein level of HDAC and acetylated histones in samples of dogs diagnosed with CTCL and compare it to normal lymphoid tissue, inflammatory lymphocytes and healthy skin. With this research, we proposed to investigate one of the epigenetic mechanisms proved to be involved in human cutaneous lymphoma and make the first step in recognize histone deregulation in canine CTCL.

3. Material and methods

3.1 Ethical statement

This study was approved by the Committee of Experimentation and Use of Animals in research (CEUA) by our institution (protocol number: 007973/18 and 010049/17).

3.2 Animals and tissue samples

Twenty-five paraffin-fixed formalin-embedded samples and six frozen samples of dogs diagnosed histologically with CTCL in the Veterinary Hospital “Governador Laudo Natel” of UNESP Jaboticabal during the period of 2014 until 2018 were selected for this research. For inclusion, samples were reviewed by morphology and immunohistochemistry including CD3 and CD79a and medical records had to be available for revision. Samples of dogs with CTCL were classified in the cutaneous lymphoma group (CL). We aimed to include healthy skin, normal lymph node and skin inflammation to compare histone expression. For this, eight samples of dogs diagnosed with lymphoplasmacytic inflammation in the skin composed the dermatitis group (DR), five healthy dogs’ lymph node samples were classified as lymph node group (LN) and eight samples of healthy dogs’ skin composed the normal skin group (NS). Groups LN and NS were only used for comparison of protein levels through Western Blot (WB). Healthy dogs that performed prophylactic castration had their popliteal lymph node removed and skin biopsied after obtaining written informed owner consent at our institution.

Medical records of all dogs were reviewed to obtain the time to progression (TTP) and median survival time (MST). TTP was defined as the period of time between diagnosis and date of progressive disease, while MST was defined as the period of the time between diagnosis and death or euthanasia. Deaths were confirmed by phone calls to owners and necropsies were not available to be performed.

3.3 Western Blot

For WB analysis, we selected three groups for comparison: CL (n=6), LN (n=5) and NS (n=8). The tissues were homogenized at 4°C in RIPA buffer (pH 7.4) containing 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (#P8310 - Sigma). The homogenate was centrifuged at 14 000 g at 4°C for 20 min. The protein levels from the supernatant were

determined by the Lowry method using bovine serum albumin (BSA) as a standard. The supernatant was mixed with sample buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, pH 6.8), and the mixture was boiled and subjected to SDS-PAGE analysis on 10% polyacrylamide gels. Proteins were transferred from the gel to nitrocellulose membranes. The membranes were blocked with 5% albumin for 4 h and incubated overnight at 4 °C with the primary antibodies to acetyl Histone H4 [Ac-Lys12] (1:1000, anti-rabbit, Sigma-Aldrich), HDAC1 (1:1000, anti-rabbit, Sigma-Aldrich), HDAC2 (1:2000, anti-mouse, Sigma-Aldrich), acetyl Histone H3 [Ac-Lys9] (1:1000, anti-mouse, Sigma Aldrich) and HDAC6 (1:3000, anti-rabbit, Sigma-Aldrich). GAPDH (1:1000, Cell Signaling, Danvers, MA, USA) and β-actin (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as loading control. The primary antibody was detected using peroxidase-conjugated secondary antibody and visualized using ECL reagents. Gel documentation and signal quantification were performed using the Bio-Image Analysis of Molecular Imager ChemiDoc XRS System (Bio-Rad, Richmond, CA, USA). The results were normalized using GAPDH and B-actin for H3Ac, HDAC6 and H4Ac, HDAC1, HDAC2, respectively. After all samples were quantified and for statistical analysis, the mean value was estimated for each group.

3.4 Immunohistochemical analysis

We performed immunohistochemistry for acetylated histones 3 lysine 9 (H3Ac) and 4 lysine 12 (H4Ac) and for histone deacetylate enzymes 1 (HDAC1), 2 (HDAC2) and 6 (HDAC6) in all groups (CL, DR, LN, NS). For this, paraffin-fixed formalin-embedded blocks were cut into 3 µm sections and mounted on silane-coated glass slides. Initially, slides were dewaxed and rehydrated as routinely performed and sections were subjected to antigen retrieval and primary antibody incubation as described in Table 1. For detection, we used a commercial polymer detection system (Novolink polymer DS, Leika Byosistems) in all reactions. Finally, samples were counterstained with Harris hematoxylin and mounted after dehydration in graded concentrations of xylenes and alcohols.

A sample of human tonsil (kindly provided from veterinary laboratory VETPAT) was used as the positive control for all antibodies. A negative control was obtained by replacing the primary antibodies with the dilution solution**.**

3.5 Evaluation of immunostaining and scoring system

Evaluation of immunoexpression was only performed for groups CL and DR. For this, we first confirmed nuclear expression in sections immunostained for H3Ac, H4Ac, HDAC1 and HDAC2 by visualizing of diffuse brown or brown yellow particles in the nuclei of cells. A scoring system previously described (Marquard et al., 2008) was performed based on two parameters, number of positive tumor cells and intensity of immunoreactivity. Five images at x400 magnification were randomly selected and 100 stromal cells (including tumor cells) were manually counted using the tool “Cell counter” of the Image J program. For the dermatitis group, the total number of lymphocytes and plasma cells were counted. Scores 1 to 4 were given depending on the percentage of positive cells as follows: score 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). Intensity of positive expression was given a score 0 for no positivity, score 1 for weak, 2 for moderate and 3 for strong expression. Each sample received a final score for the multiplication of the two parameters. The total score ranged from 0-12 and two categories were defined based on it: low expression (scores 0-8) and high expression (scores 9-12). Two different evaluators assessed the level of expression of all antibodies without knowing the clinical variables and when results were different, a double-headed microscope was used to establish an agreement.

For HDAC6 evaluation, a different method was used because of its cytoplasmic expression and impossibility of counting cells separately. Positive expression was characterized by the appearance of brown to yellow particles in the cytoplasm of cells. The percentage area of HDAC6+ cells staining within five images at x400 was calculated using the Image J program as previously described (Withers et al., 2019). First, the total cellular area was determined and measured for each image and non-cellular areas were excluded by manual selection.

Table 1. Antibodies used in the immunohistochemistry technique for histone and histone deacetylase expression

Antibody*	Clone	Origin	Type	Dilution	Antigenic recovery
Anti-Acetyl Histone H3 (Ac-Lys9)	AH3-120	Mouse	Monoclonal	1:100	Citrate buffer (pH 6)
Anti-Acetyl Histone H4 (Ac-Lys12)	SAB42000353	Rabbit	IgG fraction	1:500	Citrate buffer (pH 6)
Anti-HDAC1	AV38530	Rabbit	IgG fraction	1:75	EDTA[±] buffer (pH 9)
Anti-HDAC2	HDAC2-62	Mouse	Monoclonal	1:500	Citrate buffer (pH 6)
Anti-HDAC6	AV31451	Rabbit	Isolated	1:500	Citrate buffer (pH 6)

*Antibodies provided from Sigma-Aldrich Company. All antibodies were incubated for 2 hours.

[±]Ethylene-diamine-tetraacetic acid

After this, a positive area was manually selected and “thresholded” to highlight only the positive cells. The area selected was measured and the result was divided by the total cellular area of that image and then multiplied by 100. Thus, a percentage was calculated for each image. A final percentage was estimated from the five images for each sample. After this, low and high expression of HDAC6 was defined as values above or below the median of the percentage of positive cells.

3.6 Statistical analysis

Level of proteins defined by WB were compared through one-way ANOVA and Tukey test among groups CL, LN and NS. Scores defined by immunohistochemistry were first categorized in “Low” or “High” depending on the antibody expression and were compared by Mann-Whitney test between groups CL and DR. For HDAC6, the t-test was performed instead of the Mann-Whitney test. In the CL group, comparison among expression of antibodies were compared by Chi-squared test and then, multivariate techniques were used to find new associations using the score values. Spearman correlation test was calculated in order to associate acetylated histones and HDACs.

For multivariate analysis the scores of all 5 antibodies were initially included, but after cluster analysis only H3Ac, H4Ac, HDAC2 and HDAC6 values were selected. First, a hierarchical cluster analysis was used for establishing two different subgroups (A and B). After this, a principal component analysis was performed in order to obtain the interaction between subgroups (defined by the cluster analysis) and potential variables called principal components (PC). The number of calculated PC corresponded to the two auto-values designated as Factor 1 and Factor 2. Finally, an exploratory factors analysis was performed to identify the processes that would explain the total variance of data. Only auto-values higher than one unit (Kaiser criteria) was considered to obtain the factors. Variables that presented absolute loading larger than 0.5 and rotation of varimax factors were considered as relevant. Wilcoxon test was used for comparing the factors between subgroups.

Survival time and time to progression curves were estimated and compared between the “Low” and “High” expression groups of each antibody and between the subgroups A and B by the Kaplan–Meier method and Log-rank test. Statistical analysis was performed using GraphPad Prism and SPSS Statistics 25. For all analyses, a p-value less than 0.05 was considered significant.

4. Results

4.1 A histone profile exists in samples of CTCL in dogs

We detected a histone profile by immunohistochemistry in samples of cutaneous lymphoma in dogs. Protein identification of histones and HDACs were evident by immunohistochemistry and validated by WB. The level of proteins varied among normal lymph nodes between immunohistochemistry and WB and an increased immunohistochemical expression of H4Ac and HDAC2 were observed in 86% and 100%, respectively of samples as will be described in the subsequent segments.

4.2 Expression of acetylated histones and HDAC enzymes in cutaneous lymphoma, inflammatory cells, normal lymphoid and epithelial cells

According to WB results, lymphoid malignant cells presented different levels of acetylated histones and HDAC enzymes. Based on immunohistochemistry, the H3Ac, H4Ac, HDAC1 and HDAC2 were localized in the nuclei of cells, showing a nuclear immunostaining as illustrated in Figure 1. H3Ac showed a strong immunolabeling in the tangled threads of lymphocytes with mitotic figures (Figure 1a). Activated lymphocytes and plasma cells observed in the dermatitis group and normal lymphocytes in the lymph node group expressed also nuclear staining for the antibodies recently described.

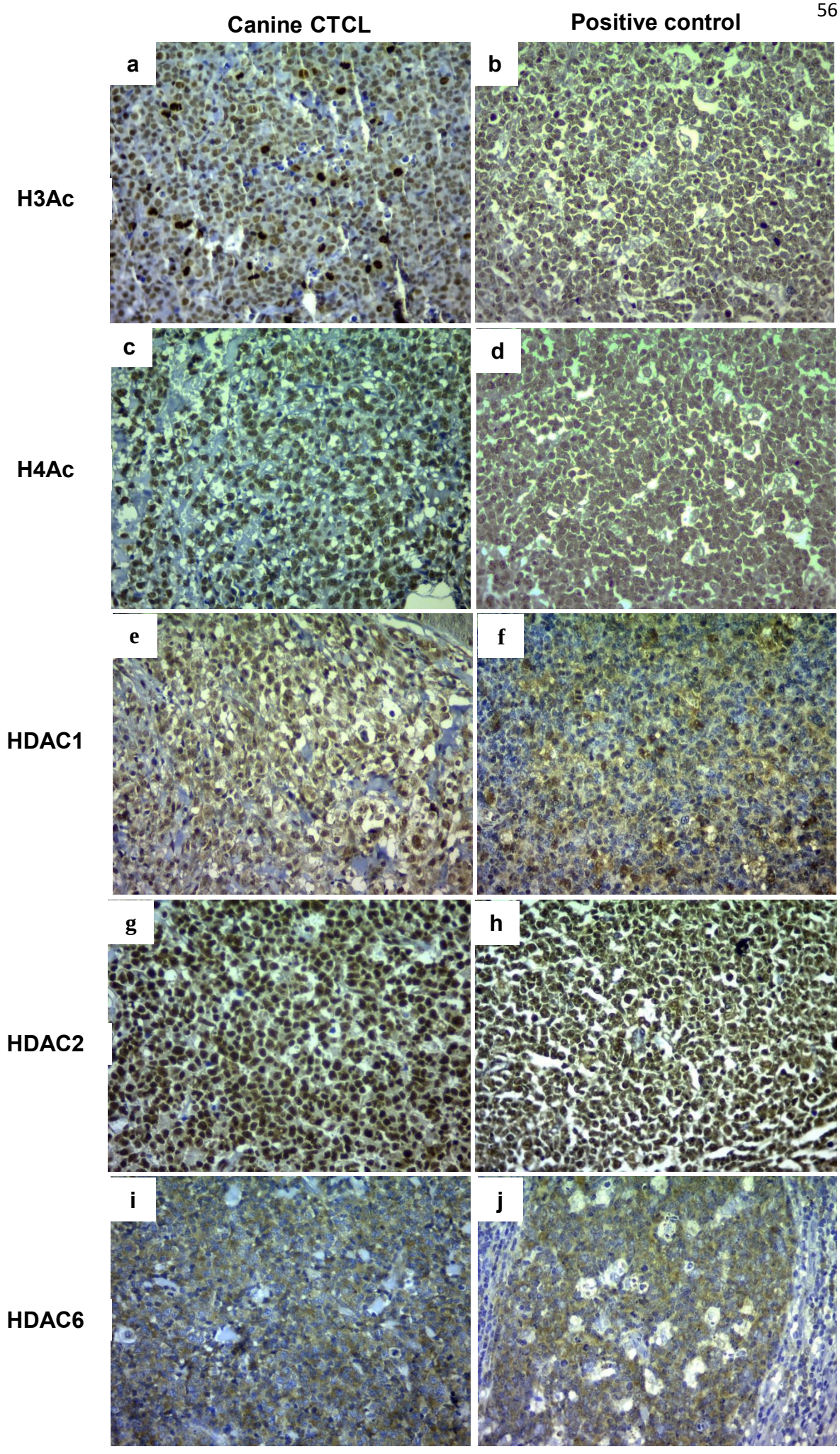
Canine CTCL
Positive control
a
b
H3Ac
c
d
H4Ac
e
f
HDAC1
g
h
HDAC2
i
j
HDAC6

Figure 1. Comparative photomicrographies of histones H3Ac and H4Ac and histone deacetylate HDAC1, HDAC2 and HDAC6 in tissues of canine cutaneous T-cell lymphoma (CTCL) (left) and human tonsil as a positive control (right). Photomicrography representative of nuclear immunostaining of H3Ac (a) H4Ac (c), HDAC1 (e) and HDAC2 (g) in CTCL and in human tonsil (b, d, f, h, respectively). HDAC6 presented cytoplasmic immunolabeling in CTCL (i) and human tonsil (j). Mitotic figures of CTCL presented darker brown staining for H3Ac (a). Immunohistochemistry reaction (x40).

In contrast to nuclear immunostaining, HDAC6 showed cytoplasmic immunoexpression of cells (Figure 1i). Epithelial and adnexal cells in the dermis showed negative expression to HDAC6. As a common characteristic, small lymphocytes in samples of cutaneous lymphoma were negative for all antibodies (Figure 2).

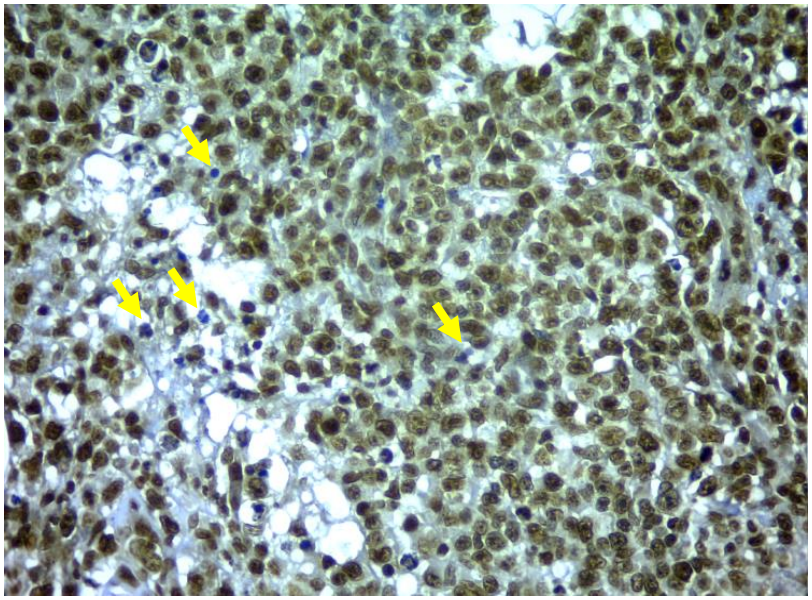

Figure 2. Photomicrography of HDAC2 immunoexpression in a sample of cutaneous T-cell lymphoma. Positive immunoexpression is seen in almost all malignant lymphocytes, however a negative immunoexpression is seen in small lymphocytes (yellow arrows) (x40).

Human tonsil lymphocytes showed a positive expression in the nuclei for H3Ac, H4Ac, HDAC1 and HDAC2, whereas a cytoplasmic immunostaining was observed for HDAC6. Thus, an identical immunolabeling of the studied antibodies in the human and

canine tissue, confirmed the immunoexpression of malignant lymphocytes in cutaneous lymphoma.

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4.3 Level of acetylated histones and HDAC in samples of CTCL

In WB results, we observed a high level of H4Ac that was statistically different (p=0.02) from the other proteins. H3Ac presented the lowest value and among the HDACs, none of them presented any difference (p>0.05). Figure 3 shows expression of the 5 antibodies by WB. The results of WB were validated by immunohistochemistry.

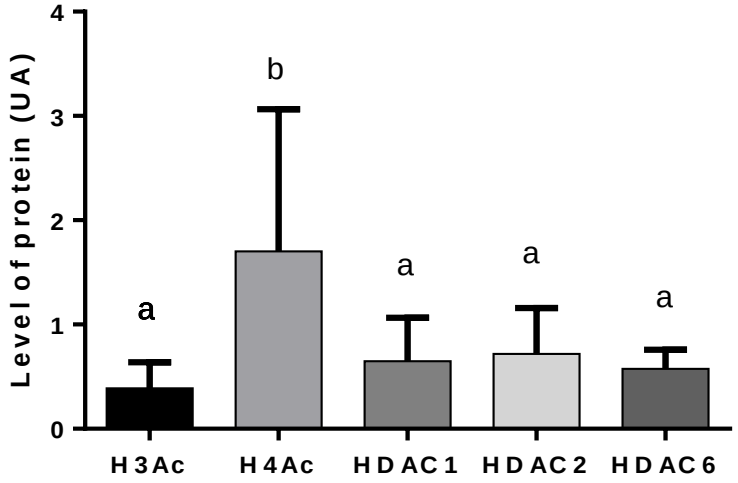


Figure 3. Level of histones (H3Ac and H4Ac) and histone deacetylases HDAC1, HDAC2 and HDAC6 in samples of canine cutaneous T-cell lymphoma by Western Blot. A high level of H4Ac and low level of H3Ac were observed in both techniques. Among HDACs, none of them presented a statistically difference. Different letters mean statistical differences among antibody levels (p<0.05).

In immunohistochemistry, samples with low and high expression were determined for antibodies according to the scoring system ranging from 0-12. All samples in CL group were positive to the studied antibodies, then a score 0 was not detected. The median value of HDAC6 was used as cutoff point to separate samples with low and high expression. We observed a strong positive expression of H4Ac and HDAC2 when compared to the other antibodies and a low expression of H3Ac.

The immunoexpression profiles were high for H4Ac and HDAC2 and in contrast, low for H3Ac, followed by HDAC1 ($p<0.0001$). HDAC6 presented medium expression. Thus, similar expression profiles were found for H3Ac and HDAC1 ($p>0.05$) and for H4Ac and HDAC2 ($p>0.05$). Whereas a significant difference was found between antibodies H3Ac and H4Ac ($p<0.01$), H3Ac and HDAC2 ($p<0.0001$), HDAC1 and H4Ac ($p<0.01$), HDAC1 and HDAC2 ($p<0.0001$) and HDAC2 and HDAC6 ($p<0.01$) (Figure 4).

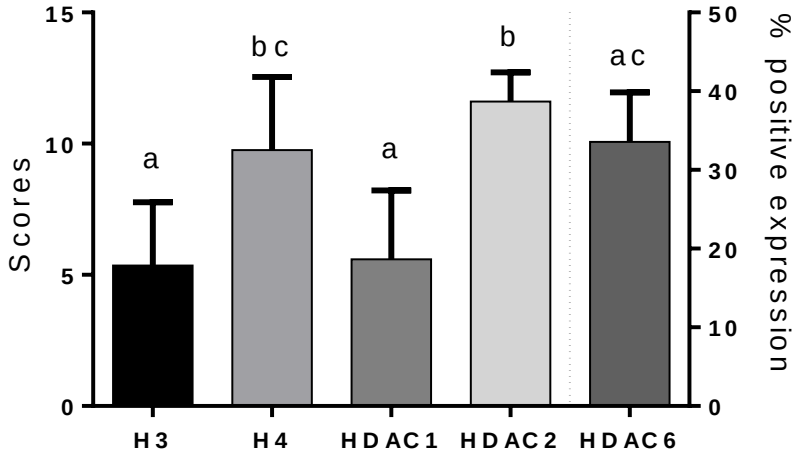


Figure 4. Immunoexpression of histones (H3Ac and H4Ac) and histone deacetylases HDAC1, HDAC2 and HDAC6 in samples of canine cutaneous T-cell lymphoma by immunohistochemistry. A different counting method was used for HDAC6 (right axis). Different letters mean statistical differences among antibody levels ($p<0.05$).

We also wanted to correlate the immunoexpression of the acetylated histones and the HDAC enzymes in order to find any association between acetylation and deacetylation, however, no correlation was found among antibodies after Spearman correlation test (data not shown).

4.4 Comparison of levels of acetylated histones and HDAC in cutaneous lymphoma, inflammatory cells, normal lymph node and epithelial cells

Normal epithelial cells presented low levels of all the proteins, which were statistically different from higher values of samples of cutaneous lymphoma or normal

lymph node cells (Table 2). Between groups CL and LN, only the level of H3Ac was found different ($p<0.001$). Figure 5 illustrates the levels of the markers among the three different groups by WB.

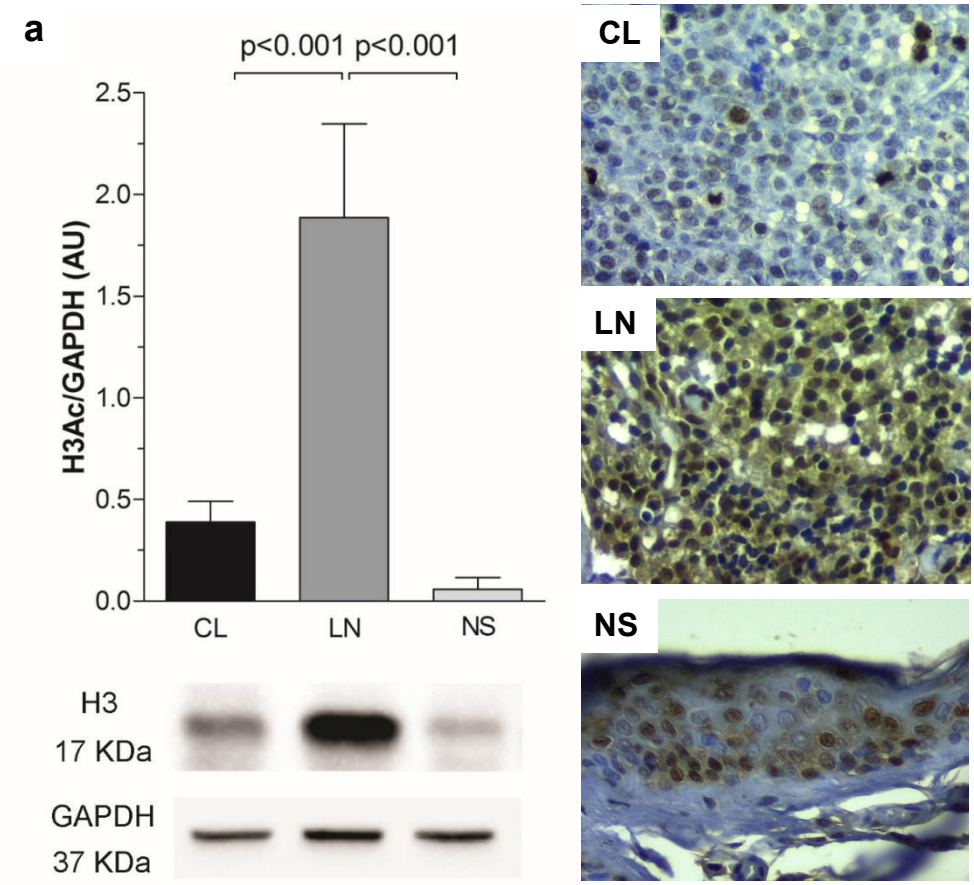
After validation of protein expression by WB, we wanted to compare through Mann-Whitney test the immunoexpression of the histones and HDAC between malignant lymphocytes and reactive lymphocytes in inflammation. Thus, we compared the scores and percentage of immunolabeling between groups CL and DR. In the statistical analysis, no difference was found for histone H3Ac and HDAC2. In contrast, H4Ac, HDAC1 and HDAC6 presented statistical difference. For HDAC6, we observed a higher immunoexpression in malignant lymphocytes (CL) than in inflammatory cells (DR), while for H4Ac and HDAC1 we observed the opposite. Figure 6 illustrates the immunoexpression among groups and their differences.

Table 2. Results of ANOVA for the three groups (cutaneous lymphoma, dermatitis and normal skin) based on the level of proteins by Western Blot.

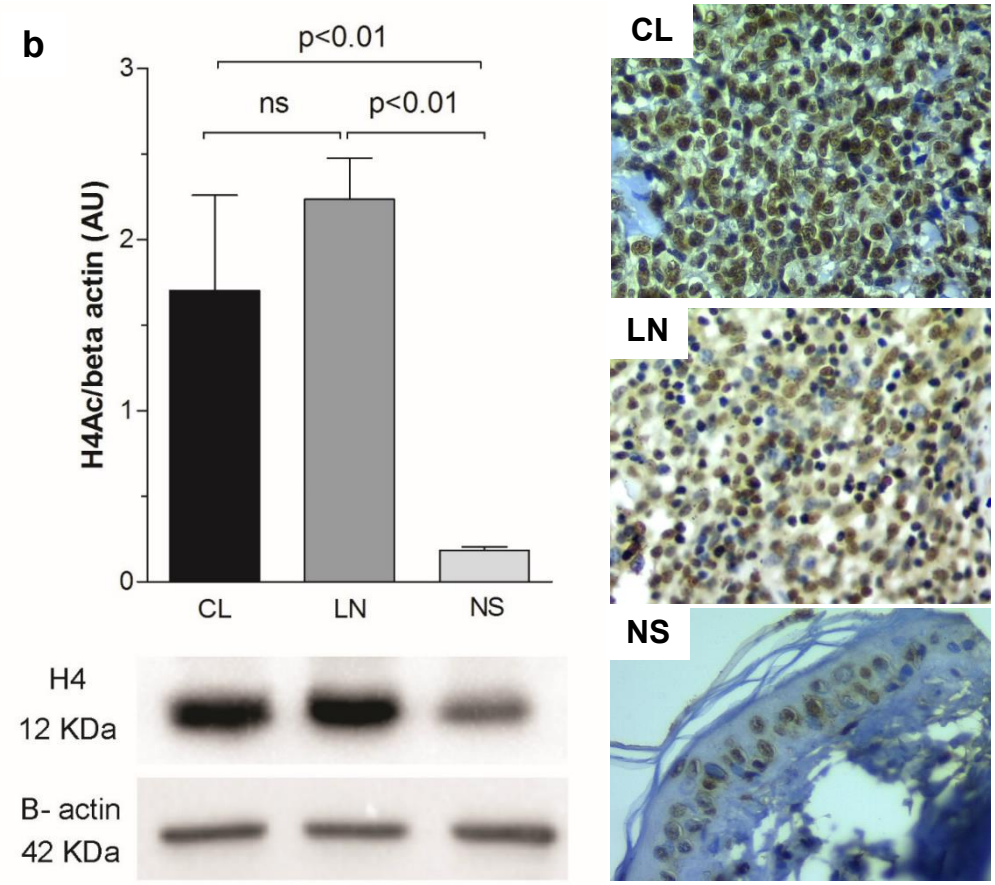
Protein	Group	n	Mean	SD	F	p value
H3Ac	CL	6	0.38	0.25	18.25	<0.0001
	LN	5	1.88	1.03		
	NS	8	0.05	0.16		
H4Ac	CL	6	1.7	1.36	10.54	0.001
	LN	4	2.23	0.47		
	NS	8	0.18	0.06		
HDAC1	CL	6	0.64	0.41	5.18	0.01
	LN	5	0.65	0.52		
	NS	8	0.12	0.04		
HDCA2	CL	6	0.71	0.44	9.86	0.001
	LN	5	0.51	0.28		
	NS	8	0.05	0.01		
HDAC6	CL	6	0.57	0.18	12.35	0.0006
	LN	5	0.37	0.37		
	NS	8	0.01	0.02		

SD: Standard deviation, HDAC: histone deacetylase, CL: cutaneous lymphoma, DR: dermatitis, LN: lymph node

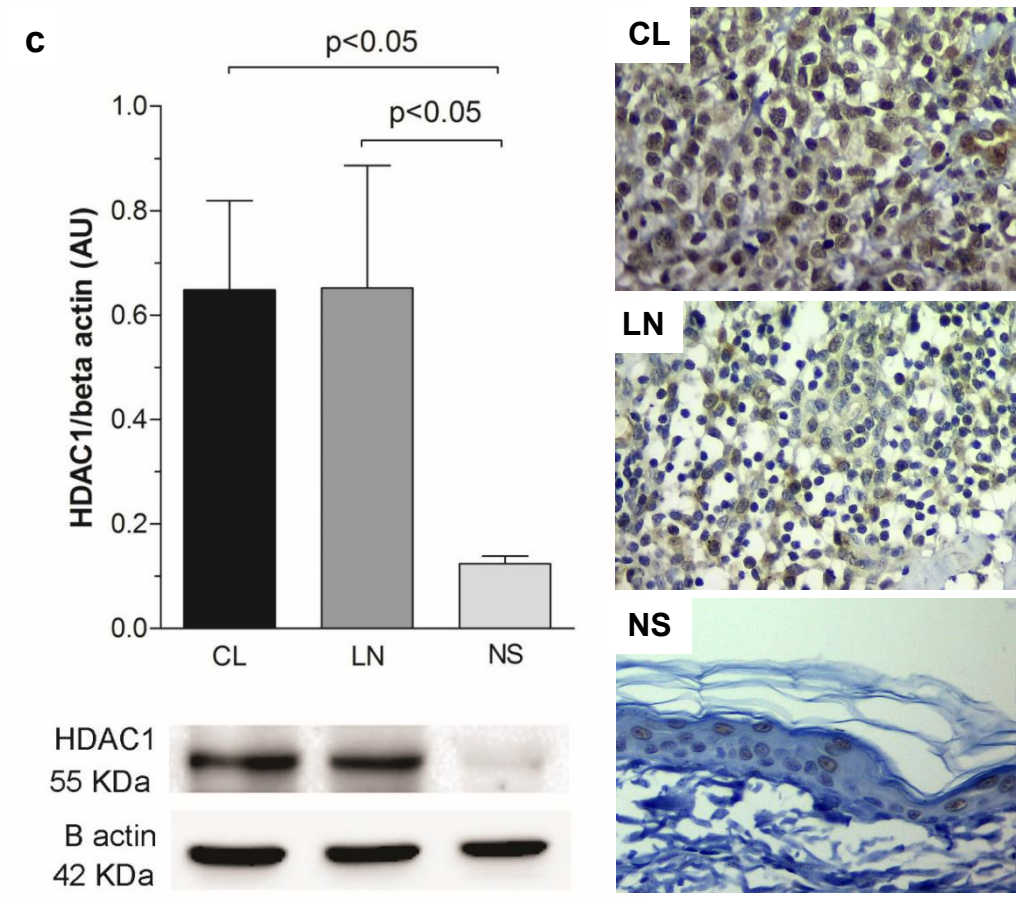
H3Ac


a
p<0.001 p<0.001
H3Ac/GAPDH (AU)
2.5
2.0
1.5
1.0
0.5
0.0
CL
LN
NS
H3
17 KDa
GAPDH
37 KDa
CL
LN
NS

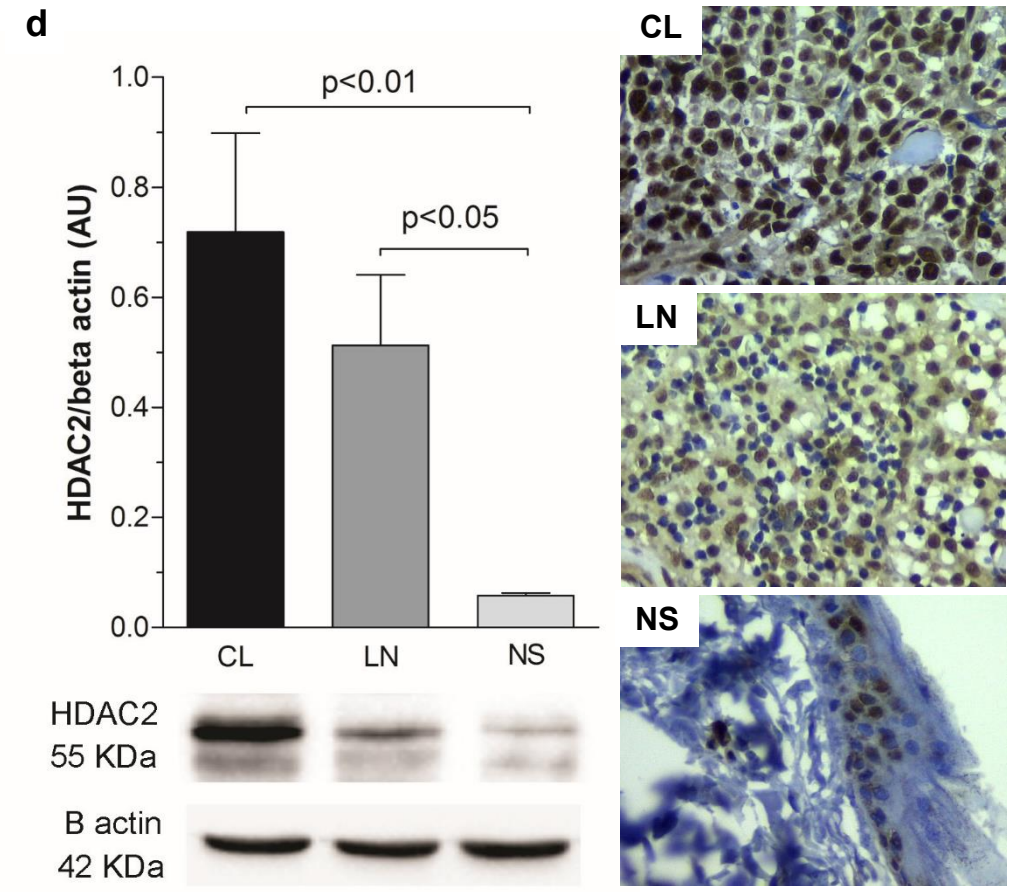
H4Ac


b
p<0.01
ns
p<0.01
H4Ac/beta actin (AU)
3
2
1
0
CL
LN
NS
H4
12 KDa
B- actin
42 KDa
CL
LN
NS

HDAC1


c
p<0.05
p<0.05
HDAC1/beta actin (AU)
1.0
0.8
0.6
0.4
0.2
0.0
CL
LN
NS
HDAC1
55 KDa
B actin
42 KDa
CL
LN
NS

HDAC2


d
p<0.01
p<0.05
HDAC2/beta actin (AU)
1.0
0.8
0.6
0.4
0.2
0.0
CL
LN
NS
HDAC2
55 KDa
B actin
42 KDa
CL
LN
NS

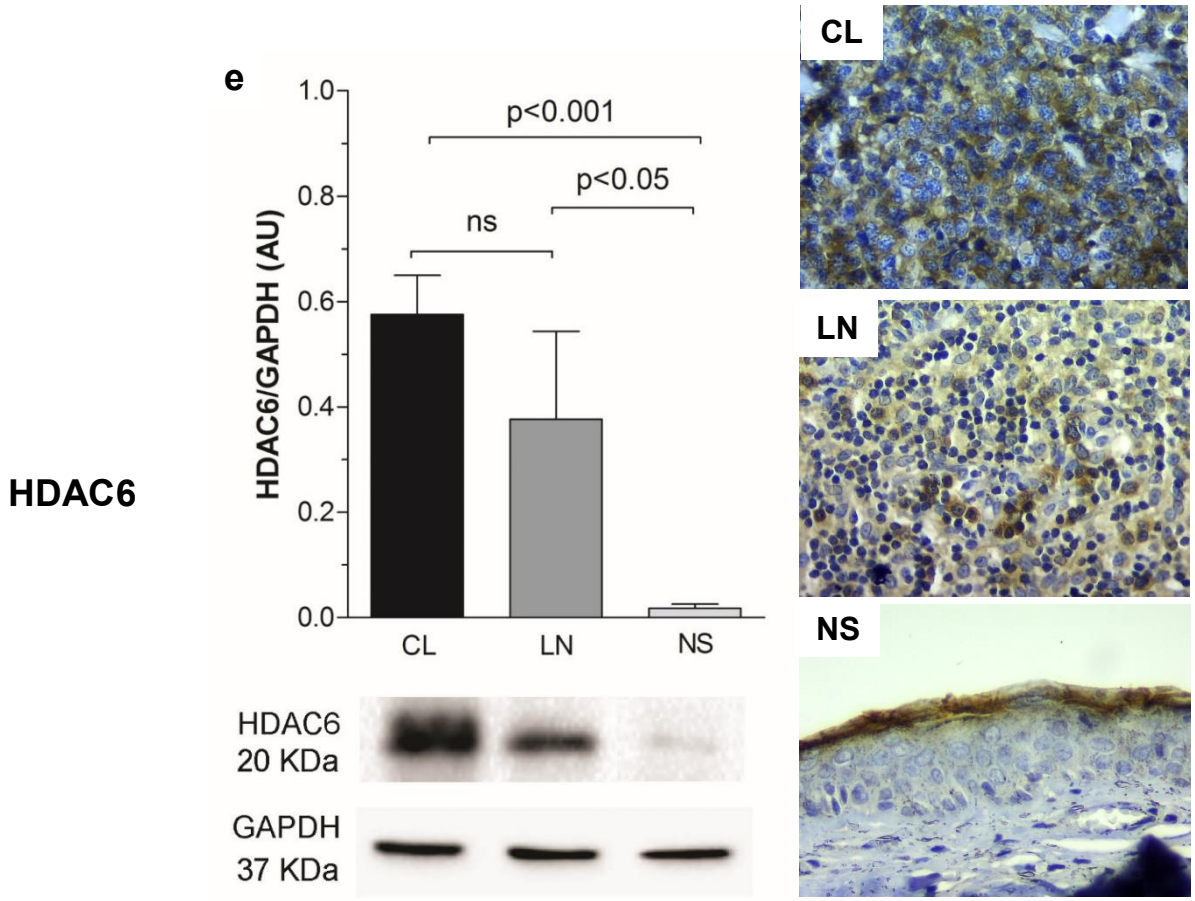


Figure 5. Western blots of levels (in columns) of histones H3Ac (a), H4Ac (b) and histone deacetylases HDAC1 (c), HDAC2 (d) and HDAC6 (e) in samples of cutaneous lymphoma (CL), lymph node (LN) and normal skin (NS). Representative images by immunohistochemistry illustrate the location and intensity of the same protein in the three different groups. Immunohistochemistry reaction (x40).

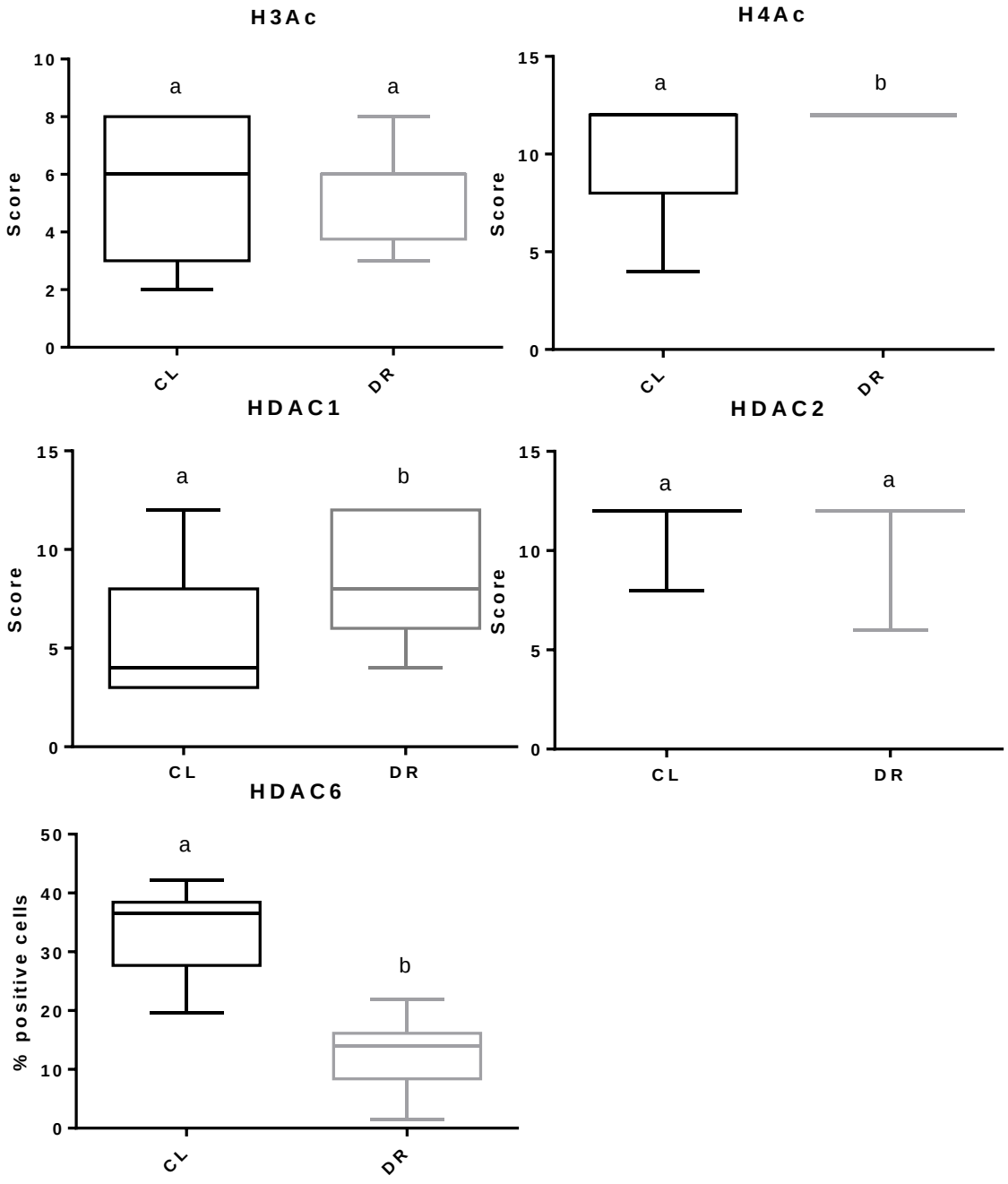


Figure 6. Level of histones (H3Ac, H4Ac) and histone deacetylases HDAC1, HDAC2 and HDAC6 in terms of immunoexpression in groups cutaneous lymphoma (CL) and dermatitis (DR). Level of expression of H4Ac and HDAC2 are showed in score, whereas HDAC6 levels mean percentage of positive cells. Different letters mean statistical differences among antibody expression ($p<0.05$).

4. 5 An aberrant modification pattern involved the immunoexpression of H3Ac, H4Ac with HDAC2 and distinguished two populations with different prognosis in dogs with CTCL

Multivariate analysis demonstrated that our samples could be classified in two subgroups (A and B) that expressed a different association pattern of histone acetylation and deacetylation. For this, we first separated the whole population of dogs from group CL into two subgroups (A and B) through the hierarchical analysis and observed that the subgroups A and B were strongly separated with a value close to 12 units of linkage distance (Figure 7). After this, we observed through a principal component analysis that the association of variables H3Ac, H4Ac, HDAC2 and HDAC6 (no influence of HDAC1) explained the formation of the subgroups A and B (Figure 8). With this information, we performed an exploratory factor analysis and identified that estimated Factor1 and 2 explained 66% of the total variance (Table 3). The association of H3Ac, H4Ac and HDAC2 constituted Factor1 – defined as aberrant histone modification – while HDAC6 constituted Factor2 – defined as histone deacetylation 6. Finally, we proved that the value of aberrant histone modification (factor1) was significantly different between subgroups A and B ($p<0.0001$), thus, confirming that a real dichotomization of samples with CTCL exist and is based on the associate expression of histones and enzymes. Values of Histone deacetylation 6 were not different between subgroups ($p=0.7$) (see Table 3).

Finally, we wanted to know whether any difference in terms of survival proportion or prognosis were in the subgroups A and B. For this, we estimated the MST and median TTP of the two populations and observed a longer MST (48 days) and median TTP (13 days) for dogs in subgroup A when compared to MST (22 days) and median TTP (8 days) for dogs of subgroup B. Despite of prolonged median times in subgroup A, no significant differences were found after comparing MST ($p=0.06$) and TTP ($p=0.38$) curves by Log rank test (Figure 9).

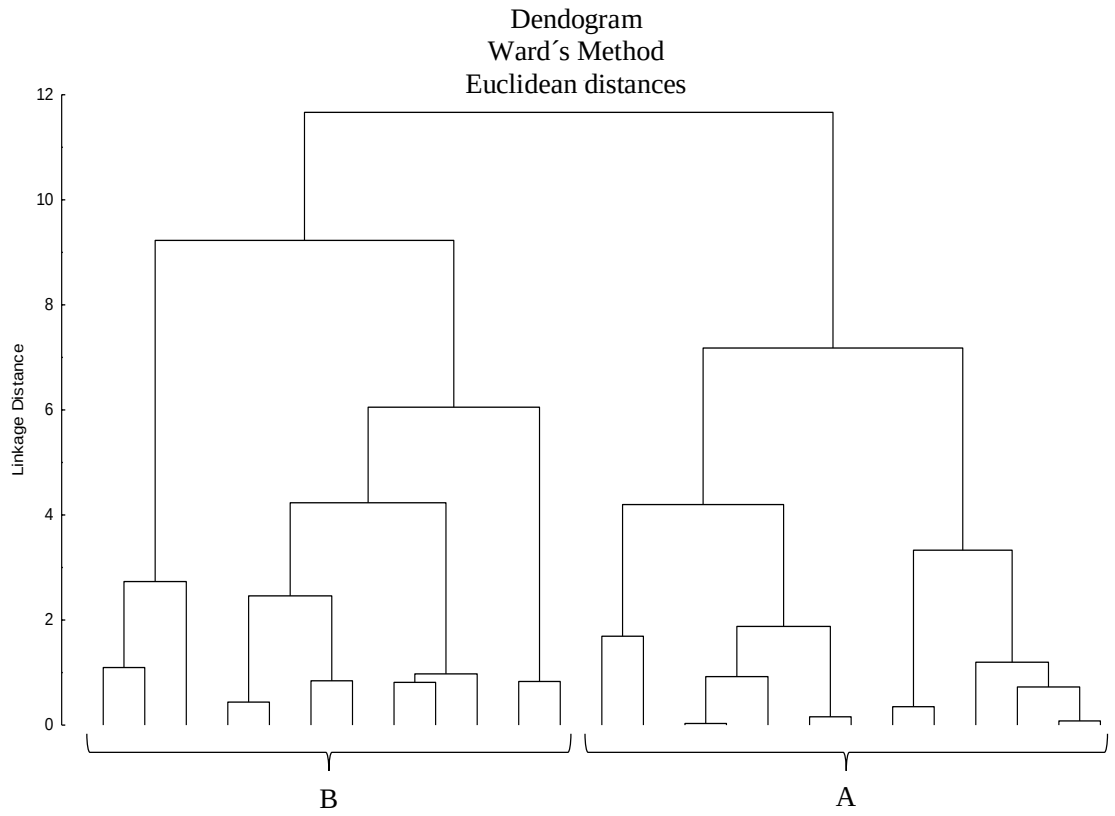


Figure 7. Illustration of the hierarchical cluster analysis based on the expression of histones H3Ac and H4Ac and histone deacetylases HDAC2 and HDAC6 in samples of dogs with cutaneous T-cell lymphoma. A clear differentiation between subgroups A and B is seen at the top of the hierarchical analysis with close to 12 units of linkage distance.

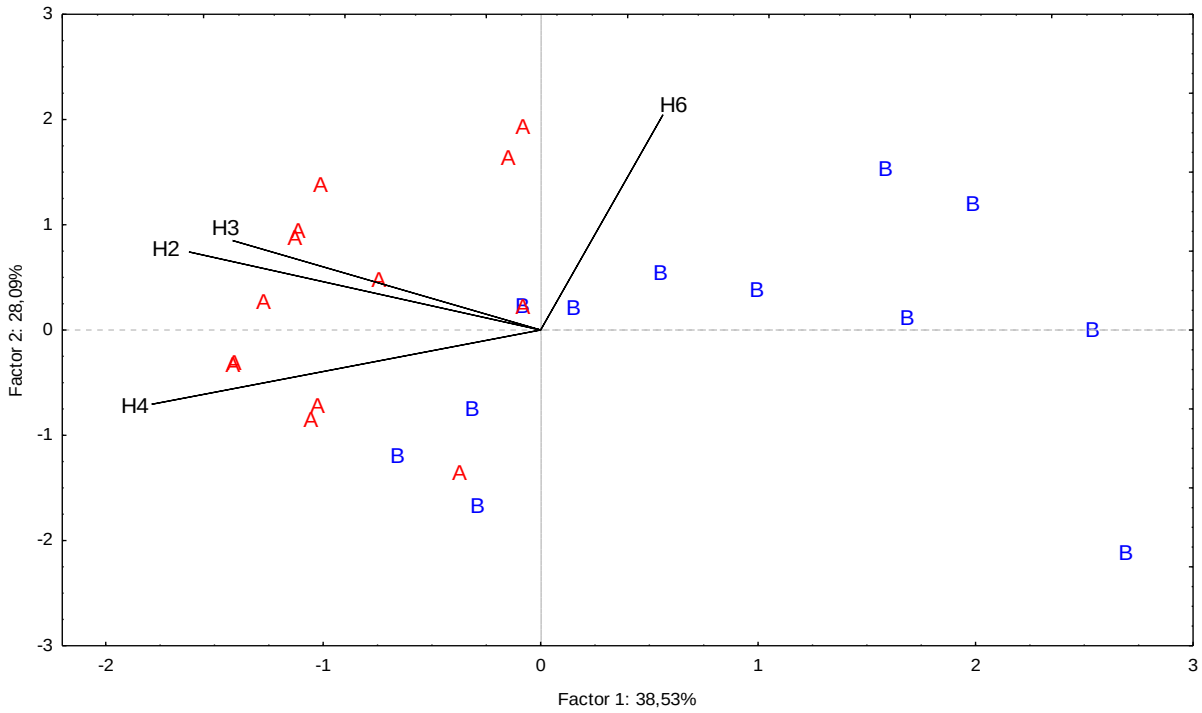


Figure 8. Biplot graph. Distribution of the variables and the subgroups A and B plotted after principal component analysis was performed. H3: H3Ac, H4: H4Ac, H2:HDAC2, H6: HDAC6.

Table 3. Results of exploratory factor analysis and Wilcoxon test. Factor1 presented statistical difference between subgroups A and B. Wilcoxon test.

	Factor1	Factor2
HDAC2	**0.77**	0.06
H3Ac	**0.70**	0.13
H4Ac	**0.62**	-0.55
HDAC6	0.07	**0.91**
Total of variance explained (%)	37	29
Interpretation	Aberrant histone modification	Histone deacetylation 6
Wilcoxon test p_value	p<0.0001	p=0.7
Median values		
Subgroup A	0.68	0.07
Subgroup B	-0.5	0.3

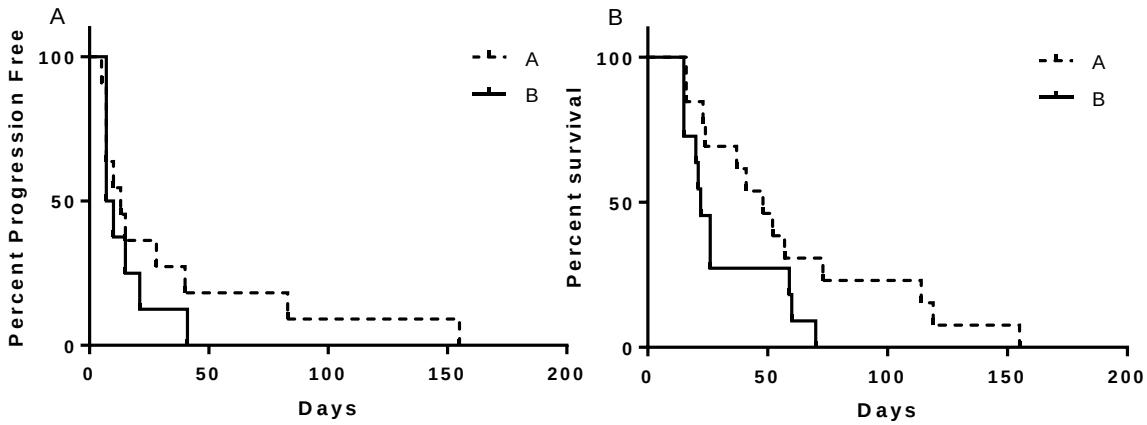


Figure 9. Time to progression and survival curves in dogs with cutaneous T-cell lymphoma based on subgroups A or B. A. Comparison of median time to progression in subgroup A (dotted line, n=10, median of 13 days) showed no significantly difference when compared to dogs of subgroup B (solid line, n=9, median of 8 days) (p=0.38). B. Comparison of median survival times in subgroup A (dotted line, n=13, median of 48 days) showed no significant difference when compared to dogs of subgroup B (solid line, n=11, median of 22 days) (p=0.06). Log Rank test.

4.6 Dogs with high immunoexpression of H3Ac lived longer than dogs with low expression

For survival analysis, we compared dogs with high and low expression seen on immunohistochemistry of the acetylated histones (H3Ac, H4Ac) and HDAC1 and HDAC6, excluding HDAC2 since all samples presented only high expression. One dog was censored of the analysis since it died 5 days after diagnosis of acute renal injury not associated to cutaneous lymphoma. No difference in TTP curves were observed for any variable. However, we observed that limiting the survival time up to 150 days (one dog censored) a statistical difference (p=0.035) was achieved in the MST of dogs with low expression of H3Ac (23 days) when compared to dogs with high expression of H3Ac (52 days) (Figure 10).

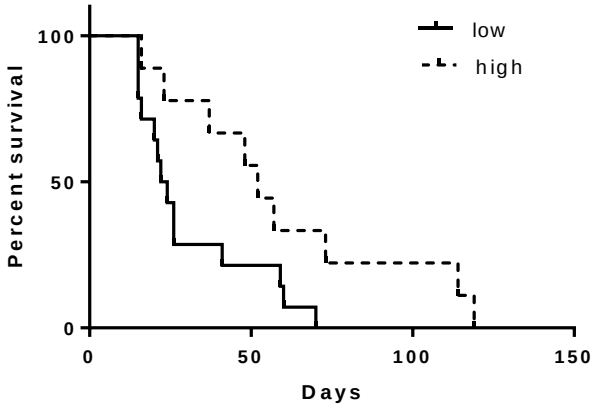


Figure 10. Survival curve in dogs with cutaneous T-cell lymphoma based on the high or low expression of the acetylated histone 3 (H3K12Ac). Patients with high expression presented longer median of survival time (dotted line, n=9, median of 52 days) when compared to dogs with low expression (solid line, n=14, median of 23 days). This difference was statistical significant (p=0.035). Log Rank test.

5. Discussion

To the best of author´s knowledge, this is the first study published of histone modifications in dogs with CTCL. We showed that histones and HDAC enzymes are present in different levels in malignant lymphocytes and that a modification pattern exists in canine CTCL through the association of HDAC2, H3Ac and H4Ac. This pattern of association defined by immunohistochemistry separated two populations with different prognosis. Different histone modification patterns have been already described in human CTCL and several other solid tumors (Zhang et al., 2005; Marquard et al., 2008; Weichert et al., 2008; Hayashi et al., 2010). In humans, different combinations of post-transcriptional histone modifications may influence how the chromatin is decodified, leading to distinct functions and processes of the cells in a process called the “histone code” (Strahl and Allis, 2000; Jenuwein and Allis, 2001). These chromatin-related altered functions may come as an epigenetic regulation of normal cells, but also, it has associated with several cancers, including CTCL (Sharma et al., 2010; Fullgrabe et al., 2011).

The understanding of the expression of histone acetylation and deacetylation can result in the translation of therapies from humans to dogs since canine CTCL is considered to have a poor prognosis as it was seen in our study. The MST of the studied population was about one month, which is shorter than previous reported survival times that ranges between 6 to 9 months (Williams et al., 2006; Risbon et al., 2006; Chan et al., 2018).

Detection of specific histone modifications by WB and immunohistochemistry constitute one of the methods to determine protein acetylation or methylation (Zhang et al., 2004). We selected antibodies for detecting acetylation at specific sites of histones 3 and 4 and for identifying the expression of deacetylate enzymes. We observed that all proteins were present in samples of CTCL. By immunohistochemistry, we confirmed nuclear immunostaining of malignant lymphocytes for H3Ac, H4Ac, HDAC1 and HDAC2. Whereas a predominant cytoplasmic immunostaining was seen for HDAC6. This nuclear and cytoplasmic preference of staining is a common and typical pattern of immunoexpression for the described antibodies in multiple human studies on multicentric and cutaneous lymphoma, as well as solid tumors (Seligson et al., 2005; Mohamed et al., 2007; Min et al., 2012; Adams et al., 2010; Lee et al., 2014). Interestingly, we observed a strong nuclear staining for H3Ac in lymphocytes expressing mitotic figures. This phenomenon can be explained by the pure nature of this histone, since histone 3 acetylated at lysine 9 is considered a mark of active chromatin and H3K9Ac is enriched at regions close to transcriptional start sites (Fullgrabe et al., 2011). One unique study evaluated H3Ac by immunohistochemistry in samples of canine tumors showing nuclear immunostaining as here described, however, authors did not describe what tumors types were stained (Wittenburg et al., 2010). Another interesting feature was the predominantly negative immunostaining of small-sized lymphocytes seen in our study and that has been reported previously in samples of CTCL in humans (Marquard et al., 2008).

One of the purposes of our study was to look for differences in the protein level of cutaneous lymphoma, dermatitis, normal lymphocytes and healthy epithelial cells. We demonstrated that the level of proteins was similar for malignant lymphocytes and normal or reactive lymphocytes for almost all markers. Still, a significant increase of H4Ac and a decreased of H3Ac were found in samples of CTCL. H3 is expected to be

hypoacetylated in tumors when compared to normal tissues (Sharma et al., 2010). Hypoacetylation of H3 at lysine 9 has been correlated to tumor progression in ovarian and prostate carcinomas (Mohamed et al., 2007; Zhen et al., 2010) and during the process of deacetylation of a residue at a specific point, a possible methylation can occur simultaneously (Fullgrabe et al., 2011). The concomitant hypoacetylation and trimethylation of H3 at lysine 9 leads the cell to aberrant gene silencing of tumor suppressor genes, a known step in cancer progression (Sharma et al., 2010). Although, normal and tumor lymphocytes presented similar expression of H3Ac in our samples, we observed a low expression profile in dogs with CTCL, indicating hypoacetylation. This low expression was associated with shorter survival time, thus confirming that hypoacetylation of H3K9 is related to worse prognosis in dogs with CTCL. Still it is unclear if hypoacetylation occurs alone or simultaneously with trimethylation, so further studies are needed to identify the methylation status or the role of methyl transferases in CTCL.

In contrast to H3Ac, H4Ac was found to be statistically higher in samples of CTCL. H4 can be acetylated at various residues including lysine 5, Lys8, Lys12 and Lys16 (Fraga et al., 2005). A loss of acetylation at Lys16 was established as a common characteristic of many human malignancies through the evaluation of different tumor types and this process occurs slowly through tumor progression and in a nonrandom manner, thus, initial acetylation is expected at Lys 16, followed by Lys12, Lys 8 and finally by Lys5 (Fraga et al., 2005). As we evaluated H4 at Lys12 rather than Lys16, we are unable to determine whether a loss of acetylation had started at Lys16 and then in a final stage of tumor progression could occur at Lys12. Another possible explanation of high H4 acetylation status is related to a loss of function of HDAC enzymes, more specifically HDAC1 (Marquard et al., 2008). The latter enzyme appeared to regulate H4 acetylation and it seemed to be dysfunctional in chronic leukemia, then hypoacetylated H4 could be the result of a tumor effect more than a cause of tumorigenesis (Brusa et al., 2006). In solid tumors, hypoacetylated H4Ac at Lys12 was considered a predictive factor for tumor recurrence in lung and prostate carcinomas (Seligson et al., 2005; Barlesi et al., 2007), but in human B or T-cell lymphoma and CTCL H4Ac was highly expressed, however the mechanism to explain this contrasting level in lymphomas is still unknown (Marquard et al., 2008; Marquard

et al., 2009). Other authors compared the immunohistochemical expression of acetylated H4 at Lys12 and observed a higher expression profile in the aggressive form of CTCL when compared to the indolent form (Marquard et al., 2008). Thus, it is possible that in dogs a similar high expression pattern of acetylated H4 at Lys12 exists.

HDAC enzymes are classified in four classes (I, II, III and IV). HDAC1 and HDAC2 are part of the class I, whereas HDAC6 is classified in the class II (Chen et al., 2015). Although similar functions and expression can be expected in HDAC enzymes of the same class, HDAC1 and HDAC2 have been reported in opposite expression profiles in different lymphoma types (Marquard et al., 2008; Lee et al., 2014). In our study, HDAC1 presented medium protein level in WB that was confirmed by immunohistochemistry. 60% and 40% of samples with CTCL presented high and low HDAC1 expression, respectively. This relative medium expression seems to contrast previous results in several forms of lymphoma including CTCL (Marquard et al., 2008; Marquard et al., 2009; Adams et al., 2010; Min et al., 2012) and solid tumors since overexpression of HDAC1 has been demonstrated in gastric, colorectal, prostatic and mammary carcinomas (Choi et al., 2001; Krusche et al., 2005; Ishihama et al., 2007; Weichert et al., 2008). In contrast, other authors have associated HDAC1 with a favorable prognosis in breast tumors (Zhang et al., 2005) while others found only 13% expression of HDAC1 in diffuse large B-cell lymphoma and HDAC1 gene expression was up-regulated in the indolent form of B-cell lymphoma when compared to the aggressive form (Rosenwald et al., 2002). Lack of immunolabeling – that would indicate an atypical pattern of HDAC1 expression- was associated to poor prognosis in Hodgkin lymphoma (Adams et al., 2010). Although we detected staining in some samples (40%), the aggressive behavior seen in the studied population may have an association with the lack of high expression seen in cases of human CTCL (Marquard et al., 2008). Taken together these results, we infer that HDAC1 present contrasting roles in diverse tumors, for this reason, it is important to establish a histone modification profile or a “histone code” for each malignancy, as suggested previously (Strahl and Allis, 2000).

In this study, a different pattern of expression was observed for HDAC2 when compared to HDAC1. In CTCL, malignant lymphocytes expressed a higher level of HDAC2 than HDAC1 determined by WB. This high level of protein was evident to differ

to the low level of HDAC2 in normal skin. According to immunohistochemistry, all samples of CTCL expressed consistently HDAC2, since 100% presented high immunoexpression. Overexpression of HDAC2 seemed to be a characteristic of solid and hematopoietic tumors (Zhu et al., 2004; Song et al., 2005; Wilson et al., 2006; Marquard et al., 2009; Weicher et al., 2008). Previous studies in human lymphoma, including CTCL have shown an increase expression of HDAC2 (Marquard et al., 2008; Marquard et al., 2009; Adams et al., 2010; Lee et al., 2014). These results, can lead us to the hypothesis that high expression can be a common feature of CTCL in humans and dogs as some authors have described a possible involvement of HDAC2 in the aggressive form of human CTCL (Marquard et al., 2008). This high expression can be associated to the fact that HDAC2 bind to the promoters of some important tumor suppressor genes such as p21, p27 and p57 and negatively regulate their function, then inhibition of this enzymes can lead the cell to mitotic arrest at either G1/S or G2/M phases (Li et al., 2016). Although we could not demonstrate a similar expression profile for HDAC1 and HDAC2, we suggest that enzymes from the same class can be expressed differently among tumor types, however previous results also showed differences between HDAC1 and HDAC2 expression in CTCL and in B and T-cell multicentric lymphoma in humans (Marquard et al., 2008; Lee et al., 2014).

Differently from HDAC1 and HDAC2, HDAC6 is classified in class II enzymes and is located mostly in the cytoplasm of cells (Sharma et al., 2010). We confirmed this location as we found HDAC6 immunostaining in the cytoplasm of all lymphocytes evaluated. HDAC6 has been associated with acetylation of other non-histone proteins and its cytoplasmic location may be related to achieve this function (Ropero and Esteller, 2007). Deacetylation of non-histone proteins make HDAC enzymes to be exert effects on multiple physiologic functions including differentiation, apoptosis, autophagy and inflammation (Chen et al., 2015). We found a higher protein level and immunoexpression of HDAC6 in CTCL when compared to inflammatory lymphocytes or normal epithelial cells. Thus, functions related to cancer development mediated by HDAC6 that included proliferation, angiogenesis, DNA-damage response, alteration of autophagy and apoptosis may be upregulated in CTCL (Li and Seto, 2016). Despite of these associations, HDAC6 was not defined as a prognostic factor; in human CTCL, HDAC6 presented a positive influence on survival times (Marquard et al., 2008).

Because validated levels of proteins were achieved by WB and immunohistochemistry and since we observed a particular profile expression for each evaluated antibody, we wished to analyze them in conjunction; in a multivariate analysis, and found two populations of dogs with different association of histones and prognosis. Studies using multivariate estimations such as principal component and exploratory factor analyses aimed to interrogate the relationship among parameters and have been described in cancer and epigenetics (Edefonti et al., 2008; Battaglia et al., 2010). In this study, association of H3Ac, H4Ac and HDAC2 constituted a factor that we defined as “aberrant histone modification” because of their combining effect of acetylation and deacetylation in the same factor. This factor could explain why the two populations (defined as subgroups A and B) were different. Subgroup A presented a higher value and lived longer when compared to subgroup B. Although MST were not statistically different (p=0.06), we believe that the low number of animals limited the power of the p value, therefore, we suggest to further increase the population in order to validate this hypothesis. Despite this lack of statistical significance, the combination of low H3Ac and high HDAC2 resulted in pattern of deacetylation already described in human lymphoma (Marquard et al., 2009; Lee et al., 2014). An aberrant increased expression of H4Ac was also associated to this pattern and although an explanation for this relationship is uncertain, previous results described a high expression of H4Ac in the more aggressive form of human CTCL (Marquard et al., 2008).

Expression of HDAC6 has been reported as low in human lymphoma and may not represent a real target for inhibition when compared to other HDAC enzymes (Gloghini et al., 2009). In our study, HDAC6 represented the most important parameter for Factor2, defined as “histone deacetylation 6” because of its natural deacetylation function. Although we could not find a difference for “histone deacetylation 6” value in the two subgroups, both estimated factors (aberrant histone modification and histone deacetylation 6) were truly representative of the dichotomization of the population since explained over 60% of the total variance.

Demonstration of the existence of HDAC in canine CTCL may not only have a therapeutic implication through the recognized antagonist effect of deacetylation induced by HDAC inhibitors, but since HDAC interact other type of receptors, including retinoids receptors (Urvalek and Gudas, 2014) its use in canine CTCL should be

stimulated in further studies. In humans it was demonstrated that an epigenetic silence of the tumor suppressor gene RARβ2 is present in renal and mammary carcinomas (Wang et al., 2005; Fang et al., 2015). In these tumors, the use of HDAC inhibitors reversed this epigenetic mark via acetylation of the RARβ2, resulting in tumor regression. Interestingly, in the same study, authors found a synergistic and more efficient effect against tumor growth when retinoids were used with HDAC inhibitors (Wang et al., 2005). Although this association was not confirmed in canine CTCL until now, prospective studies using HDAC inhibitors in association with retinoids are promising.

6. Conclusion

Based on these results, we conclude that a immunoexpression profile of H3Ac H4Ac and HDAC1, HDAC2 and HDAC6 exists in CTCL. We showed that these proteins were statistically higher in CTCL than in normal epithelial cells, demonstrating a different pattern in cancer. The pattern of expression was similar between inflammatory and tumor lymphocytes for all antibodies, except for HDAC6. An acetylation aberrant pattern was observed in CTCL by the demonstration of high levels of H4Ac. By contrast, a low H3Ac levels was a common characteristic in samples of CTCL and dogs expressing high levels of H3Ac presented better survival times. An association among the immunoexpression of HDAC2, H3Ac and H4Ac could define a population with worse survival, and the use of HDAC inhibitors should be considered in dogs with this disease, however, further studies are needed to confirm our results in order to stimulate the use of HDAC inhibitors drugs in canine CTCL.

7. References

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