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TAISMARA KUSTRO GARNICA

Vesículas extracelulares pequenas como marcador preditivo em cães com linfoma multicêntrico

Pirassununga
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UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

CERTIFICADO

Certificamos que a proposta intitulada "Vesículas extracelulares como potenciais marcadores preditivos e de doença residual mínima em cães com linfoma multicêntrico.", protocolada sob o CEUA nº 9827200717, sob a responsabilidade de **Heidge Fukumasu** *e equipe; Taismara Kustro Garnica* - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 19/12/2017.

We certify that the proposal "Extracellular Vesicles as potencial predictive and minimal residual disease markers in dog with multicentric lymphoma.", utilizing 40 Dogs (males and females), protocol number CEUA 9827200717, under the responsibility of **Heidge Fukumasu** *and team; Taismara Kustro Garnica* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 12/19/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 08/2017 a 07/2020 Área: Biociência Animal

Origem:	Hospital Veterinário UDCH						
Espécie:	Cães	sexo:	Machos e Fêmeas	idade:	0 a 15 anos	N:	40
Linhagem:	não definido			Peso:	1 a 25 kg		

Resumo: O linfoma e? a principal neoplasia hematopoie?tica em ca?es sendo o tipo multice?ntrico o mais comum. Apesar de responderem relativamente bem ao protocolo quimiotera?pico, a taxa de recidiva da doenc?a e? significativa levando parte dos animais a o?bito em um peri?odo de um ano. Desenvolver ferramentas que possam detectar doenc?a residual mi?nima previamente ao diagno?stico de remissa?o/recidiva e marcadores preditivos que possam avaliar a resposta terape?utica sa?o um dos maiores desafios da medicina atualmente, seja humana ou veterina?ria. Desta forma, o objetivo deste trabalho e? avaliar se ha? relac?a?o entre o tamanho e a concentrac?a?o de vesi?culas extracelulares (VE) plasma?ticas com a progressa?o da doenc?a (estadiamento), a resposta terape?utica e a recidiva, afim de potencialmente melhorar a detecc?a?o de doenc?a residual mi?nima e/ou propor um marcador preditivo de resposta ao tratamento. Para isto, sera?o coletadas amostras de plasma para o isolamento das VE em ca?es sauda?veis e outros com linfoma multice?ntrico: ao diagno?stico, durante e apo?s o protocolo de quimioterapia. As amostras sera?o coletadas previamente a administrac?a?o do quimiotera?pico a cada sessa?o do protocolo. O isolamento sera? feito pelo me?todo de ultracentrifugac?a?o e a mensurac?a?o pelo microsco?pio NanoSight. Por fim, pretende-se avaliar o perfil global de miRNAs presentes nestas vesi?culas de forma comparada. Com estes experimentos pretende-se desenvolver um me?todo que avalie a presenc?a de doenc?a residual mi?nima e determine o potencial da detecc?a?o de VE e seu conteu?do quanto a? utilizac?a?o na predic?a?o da resposta ao tratamento.

Local do experimento: FZEA-USP e UNESP

Pirassununga, 28 de maio de 2018

Profa. Dra. Daniele dos Santos Martins
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FICHA DE AVALIAÇÃO

Título: Vesículas Extracelulares Pequenas como marcador preditivo em cães com linfoma multicêntrico

Dissertação apresentada à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Mestre em Ciências do programa de Mestrado em Biociência Animal.

Data: _____/_____/_____

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Prof.____

Instituição: ___________________________Decisão: _______________________

Prof.____

Instituição: ___________________________Decisão: _______________________

Prof.____

Instituição: ___________________________Decisão: _______________________

“I dedicate this work to all human and canine patients who are fighting against lymphoma. These warriors were the main reason for this work.”

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“First, I would like to thank my supervisor professor Heidge Fukumasu for this opportunity and for trust in me and my work. You are inspiration for too many people including me. Thank you to be my father of science or how you like to say “papi” and to teach me everything I need. Sometimes you are boring (I am kidding), hahaha, but I know you try to do your best.”

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“If you want change, expand your mind. Old ways don't open new doors”

Kristen Butler

RESUMO

Garnica, T. K. **Vesículas Extracelulares Pequenas como Potencial Marcador Preditivo Em Cães Com Linfoma Multicêntrico**. 2020. 84 p. Dissertação (Mestrado em Ciências) - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

O linfoma é o tipo mais comum de malignidade hematológica canina, onde a forma multicêntrica é responsável por 75% de todos os casos. O tratamento padrão é o protocolo CHOP de 19 semanas, no qual 85% dos cães alcançam resposta completa / parcial; porém uma grande parcela recidiva a doença em um período de até um ano após o diagnóstico. A avaliação da resposta terapêutica e a compreensão dos mecanismos envolvidos no processo quimioresistência tem sido os maiores desafios do linfoma canino. Diante disso, esse trabalho foi dividido em dois estudos principais: No primeiro estudo investigamos *in vivo* o potencial de exossomos séricos e seus miRNAs como marcadores preditivos para o linfoma canino. No segundo estudo investigamos *in vitro* o papel dos exossomos derivados de células quimioresistentes em linhagens humana e canina de neoplasias hematopoiéticas. Para o primeiro estudo foram utilizados vinte e dois cães (8 em Remissão Completa e 14 em Progressão da Doença). Os exossomos isolados dos cães foram avaliados quanto ao tamanho e concentração e foi realizada uma triagem de 95 oncomir em amostras selecionadas de pacientes com RC e DP. Os pacientes com DP apresentaram maior concentração de exossomos séricos no momento do diagnóstico do que os pacientes com RC (D0, P = 0,0277). A análise da curva ROC foi significativa para a concentração de exossomos para predizer a resposta ao CHOP (AUC = 0,8076, P = 0,0203) e sobrevida global (AUC = 0,8333, P = 0,0136). O miR-205 (P = 0,0384) e o miR-222 (P = 0,0578) apresentaram maior frequência no grupo RC e o mir-20a foi mais expresso em pacientes com RC (P = 0,085), enquanto o miR-93 foi mais expresso em pacientes com DP (P = 0,09). No segundo estudo induzimos a quimioresistência utilizando o protocolo CHOP nas células 3132 (linfoma canino de células B) e células Jurkat (leucemia de células T humana). Mostramos que as células resistentes 3132-CR e Jurkat-CR têm um tempo de duplicação mais lento em comparação com suas células ingênuas respectivas. As células 3132 e 3132-CR secretaram uma maior quantidade de exossomos após o tratamento quimioterápico (P = 0,0187), porém não houve

diferença na quantidade de exossomos entre as linhagens (P = 0,7661). Houve um aumento na proliferação de células após o tratamento do exossomos em comparação ao controle para 3132 (P <0,001) e Jurkat (P <0,0035). No entanto, não houve diferença na proliferação comparando o tratamento usando exossomos derivados de células nativas e aqueles derivados de células CR para 3132 (P = 0,11) e Jurkat (P = 0,91). A proliferação celular foi maior após 78 horas de tratamento com exossomos para 3132 (P <0,001) e Jurkat (P <0,001). Em conclusão, os resultados gerados por esses estudos podem desencadear avanços na oncologia veterinária pela introdução da abordagem de biópsia líquida e pela compreensão de mecanismos de desenvolvimento de quimioresistência mediados por exossomos.

Palavras-chave: linfoma canino, vesículas extracelulares pequenas, resposta terapêutica, CHOP, marcador preditivo, quimioresistência.

ABSTRACT

Garnica, T. K. **The potential use of small extracellular vesicles as predictive marker in canine multicentric lymphoma**. 2020. 84 p. Thesis (Master in Science) – Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, 2020.

Lymphoma is the most common type of canine hematological malignancy, where the multicentric form is responsible for 75% of all cases. The standard treatment is the 19-week CHOP protocol, in which 85% of dogs achieve complete / partial response; however, a large portion of the disease relapses in a period of up to one year after diagnosis. The evaluation of the therapeutic response and the understanding of the mechanisms involved in the chemoresistance process have been the biggest challenges of canine lymphoma. Therefore, this work was divided into two main studies: In the first study, we investigated in vivo the potential of serum exosomes and their miRNAs as predictive markers for canine lymphoma. In the second study, we investigated in vitro the role of exosomes derived from chemoresistant cells in human and canine lineages of hematopoietic neoplasms. Twenty-two dogs were used for the first study (8 in Complete Remission and 14 in Disease Progression). The exosomes isolated from the dogs were evaluated for size and concentration and a 95 oncomir screening was performed on selected samples from patients with CR and PD. PD patients had a higher concentration of serum exosomes at the time of diagnosis than patients with CR (D0, P = 0.0277). The analysis of the ROC curve was significant for the concentration of exosomes to predict the response to CHOP (AUC = 0.8076, P = 0.0203) and overall survival (AUC = 0.8333, P = 0.0136). MiR-205 (P = 0.0384) and miR-222 (P = 0.0578) had a higher frequency in the CR group and mir-20a was more expressed in patients with CR (P = 0.085), while miR-93 was more expressed in patients with PD (P = 0.09). In the second study, we induced chemoresistance using the CHOP protocol in cells 3132 (canine B-cell lymphoma) and Jurkat cells (human T-cell leukemia). We have shown that resistant cells 3132-CR and Jurkat-CR have a slower doubling time compared to their respective naive cells. Cells 3132 and 3132-CR secreted a greater number of exosomes after chemotherapy (P = 0.0187), but there was no difference in the number of exosomes between strains (P = 0.7661). There was an increase in cell proliferation after treatment of the exosomes compared to the control

for 3132 (P <0.001) and Jurkat (P <0.0035). However, there was no difference in proliferation comparing treatment using exosomes derived from native cells and those derived from CR cells for 3132 (P = 0.11) and Jurkat (P = 0.91). Cell proliferation was greater after 78 hours of treatment with exosomes for 3132 (P <0.001) and Jurkat (P <0.001). In conclusion, the results generated by these studies can trigger advances in veterinary oncology through the introduction of the liquid biopsy approach and the understanding of mechanisms of development of chemoresistance mediated by exosomes.

Keywords: canine lymphoma, small extracellular vesicles, therapeutic response, CHOP, predictive marker, chemoresistance.

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1. INTRODUÇÃO GERAL

O linfoma é uma das neoplasias mais frequentes em cães. Segundo dados epidemiológicos do Brasil, o linfoma, está entre as três neoplasias de maior ocorrência na população canina (DE NARDI et al., 2004; FIGHERA et al., 2008), correspondendo a cerca de 80% das neoplasias de origem hematopoiética (SCHMIDT et al., 2013). A incidência estimada da doença é de 13 a 114 novos casos para cada 100.000 cães por ano (TESKE, 1994; DOBSON et al., 2002). Essa neoplasia se caracteriza pela proliferação clonal de linfócitos malignos tendo origem em órgãos linfoides, como medula óssea, timo, baço, fígado e linfonodos. Porém outros tecidos também podem ser afetados pela migração e instalação desses linfócitos através da corrente sanguínea (VAIL; THAMM; LIPTAK, 2019).

Aproximadamente 85% dos pacientes com linfoma apresentam a forma multicêntrica da doença, estando a maioria entre os estágios III e IV (VAIL; THAMM; LIPTAK, 2019). A forma multicêntrica é caracterizada clinicamente pela linfoadenomegalia generalizada ou localizada. O tipo não-Hodgkin (NH) é mais diagnosticado, correspondendo a 90% dos casos. Dentro do grupo dos linfomas NH, os de origem em células B são os mais comuns e apresentam comportamento menos agressivo e prognóstico mais favorável em relação aos linfomas multicêntricos de células T, que representam cerca de 10-38% dessas neoplasias (REBHUN et al., 2011) . O linfoma difuso de grandes células B é o subtipo histológico mais presente na população canina (VALLI et al., 2011).

O diagnóstico do linfoma em cães é realizado através da avaliação clínica pela citologia através da aspiração dos linfonodos ou ainda pela análise do tecido comprometido através da histopatologia (ZANDVLIET, 2016). Os cães com linfoma podem ser assintomáticos ou sintomáticos de acordo com o estadiamento da doença. Os sinais clínicos mais frequentes são linfoadenomegalia, dor, hiporexia, febre, emagrecimento e edemas. Exames de imagem como ultrassom e a radiografia, perfil hepático e renal, hemograma e o mielograma também são utilizados no diagnóstico, bem como no estadiamento do paciente (VAIL; THAMM; LIPTAK, 2019). O estadiamento do linfoma em cães segue as orientações da Organização Mundial da Saúde (OMS), sendo classificados em cinco estágios (I-V) de acordo com a localização das lesões e comprometimento de outros órgãos

e medula óssea. Eles também são subclassificados quanto à presença de alterações sistêmicas em a e b (OWEN, 1980).

O tratamento preconizado para o linfoma multicêntrico canino é a quimioterapia. O protocolo “CHOP” é a primeira escolha terapêutica para o tratamento de linfomas NH de qualquer imunofenótipo (células B e T) em cães (REBHUN et al., 2011) . A sigla, em inglês, refere-se aos quatro medicamentos que compõe esse protocolo, sendo a letra C correspondente a ciclofosfamida, a H a hidroxidoxorubicina, O ao Oncovin® (Vincristina) e o P a prednisona (REBHUN et al., 2011; MOORE, 2016). Esse protocolo alcança uma taxa de remissão completa de 85% após o tratamento e os cães tratados alcançam uma sobrevida média de 8 a 12 meses. Entretanto, o CHOP não possui um efeito curativo, o que resulta no relapso e morte de cerca da metade dos cães tratados no período de até um ano após o dignóstico e apenas um quarto dos pacientes se mantém em remissão por um período de até dois anos (BURTON; GARRETT-MAYER; THAMM, 2013). Esses dados mostram que há respostas terapêuticas distintas entre pacientes com o mesmo diagnóstico e que a recidiva é um fator importante estando diretamente relacionado à morte pela doença.

O alto índice de recidiva pós tratamento pode ser explicado pela indução de mecanismos de quimioresistência nas células neoplásicas, como a expressão dos genes relacionados a quimioresistência a múltiplas drogas, como o MDR em inglês *“multi-drug resistance”* (LEE et al., 1996). Outro fator relacionado a recidiva é o método de diagnóstico da remissão. Atualmente a remissão é baseada no desaparecimento dos sinais clínicos, porém esses não refletem a carga de células tumorais no paciente, o que torna esse parâmetro insuficiente para predizer a resposta terapêutica e o estado do paciente. Com isso, torna-se necessário o desenvolvimento de marcadores preditivos mais precisos que tenham uma relação direta com as células tumorais (SATO et al., 2016).

As biópsias líquidas estão ganhando atenção para monitorar e identificar a resposta terapêutica nas neoplasias hematológicas humanas (ROSSI et al., 2019). A busca de moléculas no sangue, como DNA circulante (cDNA) do tumor, vesículas extracelulares (VE) ou miRNAs, poderá melhorar o entendimento sobre as bases moleculares do linfoma e também trazer informações relevantes para ajudar na decisão clínica e de tratamento (CROWLEY et al., 2013).

As Vesículas Extracelulares (VE) ou vesículas derivadas das células possuem de 30 nm a 1µm de diâmetro e a suas funções fisiológicas mais importantes são a comunicação celular e transmissão de macromoléculas entre as células (MINCIACCHI; FREEMAN; DI VIZIO, 2015). De acordo com a Sociedade Internacional de Vesículas Extracelulares essas são classificadas em: microvesículas, exossomos, ectossomos, corpos apoptóticos, entre outros. Há diferentes tipos de vesículas descritos na literatura, sendo as nomenclaturas relacionadas à célula de origem ou a sua composição orgânica, o que gera uma certa inconsistência na classificação. Contudo, as mais conhecidas são as microvesículas e os exossomos (VAN DER POL et al., 2012). As microvesículas são fragmentos da membrana plasmática da célula liberado sob condição de estresse celular, medem de 100 a 1000 nm, sendo produzidas pela maioria das células e podem ser encontradas na maioria dos fluidos corpóreos. Os exossomos por sua vez são vesículas geradas por corpos multivesiculares ou por fusão direta à membrana plasmática, possuem diâmetro de 30 a 100 nm, são produzidas pela maioria das células de eucariotos e são encontradas em todos os fluidos corporais, como: sangue, urina e plasma (KELLER et al., 2006; VAN DER POL et al., 2012).

As VE têm demonstrado propriedades pro-tumorigênicas, ou seja, podem exercer um papel importante na progressão tumoral e na modulação de mecanismos celulares associados aos “hallmarks” (Yang & Robbins 2011; Sharma et al. 2016). Um estudo demonstrou que as células neoplásicas em cultivo secretam uma maior quantidade de exossomos se comparado a células normais (HENDERSON; AZORSA, 2012). Corroborando com as informações anteriores, alguns estudos também demonstraram que pacientes humanos diagnosticados com alguns tipos de câncer possuem um aumento da quantidade das VE circulantes no sangue em comparação a pessoas saudáveis (TAYLOR; GERCEL-

TAYLOR, 2008; LOGOZZI et al., 2009). Essas vesículas podem carrear informações importantes do seu tumor de origem, como moléculas de mRNAs, proteínas, lipídeos e miRNAs relacionados ao desenvolvimento e progressão das células neoplásicas (SHARMA; KHATUN; SHIRAS, 2016).

Os miRNAs relacionados às neoplasias ou "Oncomirs" exercem um papel fundamental na expressão de oncogenes e genes supressores tumorais participando do processo de carcinogênese (ESQUELA-KERSCHER; SLACK, 2006). Em um estudo com linfoma do tipo NH em humanos foi demonstrado que os níveis plasmáticos de miRNAs das VE refletem a presença vital do tecido tumoral, sendo capazes de monitorar de maneira individualizada a resposta terapêutica e a recidiva nos pacientes (VAN EIJNDHOVEN et al., 2016).

Diante disso o nosso trabalho teve como objetivo investigar o potencial das VE e seu conteúdo como marcador preditivo em cães com linfoma multicêntrico e também compreender a função das VE no processo de quimioresistência. Para isso o trabalho foi dividido em dois estudos. O objetivo do primeiro estudo foi avaliar uma biópsia líquida baseada em vesículas extracelulares pequenas (VEP) séricas para predição da resposta terapêutica de cães com LM à quimioterapia. No segundo estudo, avaliamos o potencial das VEP secretadas por células de linfoma quimioresistentes conduzirem alterações fenotípicas em células de linfoma não resistentes.

Contudo, os resultados gerados neste trabalho demonstraram a função das VEP na resposta terapêutica e elucidaram uma possível relação entre as VEP e os mecanismos de quimioresistência. Os resultados obtidos podem desencadear avanços na oncologia veterinária pela introdução da abordagem de biópsia líquida, auxiliando os veterinários a identificarem precocemente pacientes refratários e orientando o melhor tratamento possível, abrindo uma janela para a medicina de precisão na oncologia veterinária.

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2. ARTICLE 1:

LIQUID BIOPSY BASED ON SMALL EXTRACELLULAR VESICLES PREDICTS CHEMOTHERAPY RESPONSE OF CANINE MULTICENTRIC LYMPHOMAS

2.1. ABSTRACT

Lymphoma is the most common type of canine hematological malignancy where the multicentric (cMCL) form accounts for 75% of all cases. The standard treatment is the CHOP 19 weeks protocol where 85% dogs achieve complete/partial response; however, it is very important to predict non-responsive cases to improve treatment and to develop new targeted therapies. Thus, here we evaluate a liquid biopsy approach based on serum Small Extracellular Vesicles enriched for exosomes (SEVs) to predict cMCL outcome and chemotherapy response. Twenty-two dogs (8 Complete Remission and 14 Disease Progression) were evaluated regarding small extracellular vesicles size, concentration and a screening of 95 oncomirs was performed in selected samples from CR and DP patients. The DP patients had higher concentration of SEVs at the moment of diagnosis than CR patients (D0, P = 0.0277). The ROC curve analysis was significant for SEVs concentration to predict the response to CHOP (AUC=0.8076, P = 0.0203) and overall survival (AUC=0.8333, P = 0.0136). The caf-miR-205 (P= 0.0384) and caf-miR-222 (P=0.0578) had higher frequency in the CR group and caf-mir-20a was elevated in patients with CR (P = 0.085), while caf-miR-93 was highly abundant in patients with DP (P = 0.09). To the best of our knowledge, this is the first study to demonstrate the potential of predicting response to CHOP and outcome of lymphoma patients using a liquid biopsy based on SEVs and their miRNAs content.

Keywords: small extracellular vesicles, oncomir, CHOP, miRNA, predictive marker.

2.2. INTRODUCTION

Lymphoma is the most common type of hematological malignancy that occurs in dogs. The multicentric form accounts for 75% of all canine lymphomas and is characterized by generalized lymphadenomegaly (ZANDVLIET, 2016). Lymphoma in dogs has been recognized as good model for comparative studies since it is remarkably similar to human lymphoma (DEWEERDT, 2018). This cancer has a large heterogeneity with a variety of subtypes and DLBCL (diffuse large B-cell lymphoma) is the most common subtype for both species (MARCONATO; GELAIN; COMAZZI, 2013; ARESU, 2016). Among the subtypes, T-cell lymphomas are well recognized by poor prognosis and therapeutic response to CHOP compared to DLBCL lymphoma(SAYAG; FOURNEL-FLEURY; PONCE, 2018).

Despite the differences in diagnosis, most cases are generally treated as a single entity disease (WOLF-RINGWALL et al., 2019). The recognized standard protocol of canine lymphoma is 19-week CHOP with 85% of patients achieving complete or partial remission (MARCONATO et al., 2016; ZANDVLIET, 2016b). Although the initial favorable response, the majority of dogs generally relapse and the cure is rare(WITHROW, S J;VAIL, 2007; REGAN; KAPLAN; BAILEY, 2013). The consequences of relapse are a decrease in chances of achieve remission again and the response to secondary protocols (BURTON; GARRETT-MAYER; THAMM, 2013). Dogs that does not show an initial response to CHOP or relapse during or after chemotherapy regimen are a source of information about refractory profile and chemoresistance development (RICHARDS; SUTER, 2015; BRYAN, 2016). However, to determine which patients will respond or not to CHOP before starting the chemotherapy is a major challenge in canine lymphoma.

Liquid biopsies are gaining attention to monitor and identify therapeutic response in human's hematological malignancies. The search of molecules in blood such as tumor cDNA, exosomes or miRNAs can improve the molecular pathogenesis of lymphoma and also bring relevant information to help clinical and treatment decision. The exosome, a small extracellular vesicle (30-150 nm) with double lipidic membrane has an important role in cellular communication and transport of important biological molecules (mRNA, miRNA, metabolites, proteins, receptors) between cells (RAPOSO; STOORVOGEL, 2013). This vesicle is also

enrolled in cancer development and can be easily detect in organic fluids such as blood, urine, saliva(BOUKOURIS; MATHIVANAN, 2015; JAYASEELAN, 2019), thus eliciting exosomes as potential candidates for liquid biopsy approaches (SHARMA; KHATUN; SHIRAS, 2016). The miRNAs, small non-coding RNA molecules, carried by exosomes can be important to investigate pathways related to cancer metastasis, prognosis, therapeutic response and chemoresistance mechanisms (BOELENS et al., 2014; CAIVANO et al., 2015; SYN et al., 2016) . Karlee and collaborators analyzed 38 miRNAs and found altered expression of miR-127, miR-34a and miR125b in plasma comparing dogs with lymphoma that relapsed and healthy control dogs (CRAIG et al., 2019). An in vitro study showed three exosomal miRNAs (miR-151, miR-8908a-3p, and miR-486) and CD82 protein with different expression between vincristine-sensitive canine cancer cell lines (CLBL-1 and GL-1) and the resistant cell line (UL-1) (ASADA et al., 2019).

Although advances have been made in the field of veterinary oncology there is no test to predict the therapeutic response in canine lymphoma to the best of our knowledge. Therefore, our goal was to evaluate the potential of SEVs and its miRNAs content as predictive marker for therapeutic response and outcome of canine multicentric lymphoma.

2.3. MATERIAL AND METHODS

2.3.1. Case recruitment

Thirty healthy dogs were chosen to compose the control group and twenty-five dogs diagnosed with multicentric lymphoma were chosen according to eligible criteria. The samples were collected from January 2017 to January 2019. The control group samples were provided by Veterinary Hospital of Faculty of Animal Science and Food Engineering in Pirassununga (Pirassununga, SP, Brazil). Lymphoma samples were collected from two other veterinary hospitals: Anhembi Morumbi Veterinary Hospital in São Paulo (São Paulo, SP, Brazil) and governor “Laudo Natel” Veterinary Hospital in Jaboticabal (Jaboticabal, SP, Brazil). Informed consent was obtained from all clients and an animal utilization protocol according

to Animal Use Ethics Committee (CEUA) was approved by the Faculty Animal Science and Food Engineering (CEUA n: 9827200717). The control group was composed of prospective and retrospective serum samples from healthy dogs tested by clinical examination and confirmed by blood tests (complete blood count, alanine aminotransferase, creatinine, alkaline phosphatase, urea), without previous diagnosis of any disease (cancer, chronic inflammation, metabolic disease, reproductive disease, obesity and others) and with updated vaccination and deworming. The lymphoma samples were from prospective and retrospective cases of dogs diagnosed with multicentric lymphoma by cytological analysis. The cases were classified according to published by WHO (clinical examination, cytology, histopathology, PARR, immunohistochemistry). Only patients without previous diagnosis of cancer, treated with CHOP protocol, without concomitant diseases and did not receiving other drugs beside CHOP were selected.

2.3.2. Evaluation of therapeutical response

The response to the chemotherapy was evaluated following the criteria published before (2003) (ETTINGER, 2003). Patients were classified as complete or partial remission, disease in progression or stable disease. All patients were followed up until the end of CHOP and when available we also recorded clinical information about returns.

2.3.3. Serum samples, isolation and characterization of exosomes

The samples were collected at the diagnosis (D0) before the start of the CHOP chemotherapy protocol and for control group during the clinical examination. Exosomes were obtained from samples of D0 from lymphoma patients and control group. Upon collection, 2 ml of serum was centrifuged at 4°C in order to remove live cells (300 x g for 10 min), cellular debris (2,000 x g for 10 min) and microvesicles (16,500 x g for 30 min). The remaining supernatant was divided in 200 µl aliquots and maintained at -80°C until isolation of small EVs. On the day of use, 200 µl of serum was filtered through a 0.22 µM sterile syringe filter (PES membrane; KASVI)

in order to remove any remaining large EVs. Finally, this fluid was centrifuged twice at 120,000 x g for 70 min (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter) in order to isolate small EVs as previously described (THIERRY et al., 2006). The supernatant was discarded, and the exosome pellets were resuspended in 50μL of phosphate buffered saline (1X Ca2+/Mg2+ free PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) until further use. The exosomes isolated from canine serum were characterized based on their morphology and size using transmission electron microscopy; specific membrane proteins CD9 and Cytochrome C characterized by western blotting, and particle size and concentration was determined using nanoparticle tracking analysis (NTA). The protocols were based on previous work and briefly described above(DA SILVEIRA et al., 2017; DE ÁVILA et al., 2019).

2.3.4. Transmission electron microscopy

Exosome pellets isolated from 200 μl of serum of dogs from control and lymphoma group were diluted in 50μl of fixing solution (0.1M cacodylate; 2.5% glutaraldehyde and 4% paraformaldehyde at pH 7.2-7.4) for 2 h at 4℃. Subsequently, the exosomes were diluted in 2 mL of 1X Ca^{2+}/Mg^{2+} free PBS, and the solution was centrifuged once in order to obtain pellets of exosomes (120,000 x g, 70 min, 4°C). The pellet was diluted in 100 μL of milli-Q water and placed in a copper grid for 20 min at room temperature in order for it to dry before staining. The grid was inserted into 2% of uranyl acetate and then analyzed using a transmission electron microscope (FEI Tecnai 20; LAB6 emission; 200kV).

2.3.5. Nanoparticle tracking analysis

The exosomes isolated from 200 μL of serum from control and lymphoma group samples were resuspended in 50 μL of 1X Ca^{2+}/Mg^{2+} free PBS. The particle size and concentrations were measured using Nanosight (NS300; NTA 3.1 Build 3.1.45,

Malvern). The dilution factor was between (1:100 and 1:200) for 1X Ca^{2+}/Mg^{2+} free PBS depending on sample concentration. The analysis was performed by capturing 5 videos of 30 sec each, using a sCMOS camera at camera level 14 and under a controlled temperature of 37 °C. A threshold of 5 and a total valid track up to 2.500 were considered in this analysis.

2.3.6. Western blotting

The protein lysate from exosomes were obtained using RIPA buffer and 0,1% proteinase inhibitor cocktail (Halt™ Protease Inhibitor Cocktail (100X), Thermo Fisher Scientific, USA). Canine tissue (spleen) collected from a necropsy was used as negative control. The samples were prepared using 5 µl Laemmli and beta-mercaptoethanol 4x (Bio-rad) in 20 µL (~20µg) of protein solution. Denaturation of the proteins in these samples was accomplished by transferring the samples to 95°C for 5 min. The samples were then loaded onto SDS-PAGE 12% polyacrylamide gel. The gel was run at 100V for 140 min, and then the proteins were transferred on to a PVDF membrane (1704156; Trans-Blot Turbo; Bio-Rad; Hercules; CA, USA). The transference was run at 80V for 120 min and then, the membrane was washed in 1x Tris buffered saline with Tween-20 (TBST) and maintained in a blocking buffer (5% of bovine serum albumin (BSA) in TBST) at room temperature for 1 h. After that, the membrane was incubated overnight with a primary antibody at 4°C. The proteins CD9 and Cytochrome C were evaluated using antibodies CD9 (C-4) Santa Cruz (sc-13118) dilution (1:2000) and Cytochrome C (C-20) Santa Cruz (sc-8385) dilution (1:750). After incubation, the membrane was washed three times using 1x TBST for 5 min each and then incubated with secondary anti-mouse (1:2000; #7076S; Cell Signaling Technology) and anti-goat (1:2000, #B2709; Santa Cruz Biotechnology) for 1 h at room temperature, both antibodies were conjugated to horseradish peroxidase (HRP) for chemiluminescent detection. Finally, the membrane was washed three times using 1x TBST and exposed to a detection solution (170-5060; Clarity Western ECL). The images were obtained, and analysis was performed using the ChemiDoc MP Image System (Bio-Rad; Hercules; CA, USA).

2.3.7. Rna extraction

RNA was obtained using TRIZOL (Life Technologies, USA) with previous addiction of coprecipitate PolyAcryl (Carrier) from 5 samples of responder's group and 5 samples non-responder's group. For this assay, 8 ul of PolyAcryl and 750 μl of Trizol in 50 μl were added to exosome samples and incubated for 5 min. Then, 200 μL of chloroform were added and incubated for 3 minutes. The samples were centrifuged for 15 minutes as 12,000 x g at 4°C. The aqueous phase was mixture with 500 μL of isopropanol and incubated for 10 minutes. The samples were centrifuged for 10 minutes at 20,000 x g at 4°C. The pellet was resuspended in 1mL of 75% ethanol and centrifuged for 5 minutes at 20,000 x g at 4°C twice. Finally, the supernatant was discarded, and the pellet was resuspended in 10 μL of RNase-free water. Samples were treated using DNAse kit (Ambion, USA) according to manufacture instructions and stored in -80° freezer until use. The RNA concentration and quality were assessed by NanoDrop (Thermo Fisher Scientific, USA).

2.3.8. Real-time PCR for 95 Oncomirs

The total miRNAs were reverse transcribed using miScript II RT Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, 10 µL reactions were made, containing 50 ng of total RNA, 10x miScript Nucleic mix, nuclease-free water, miScript reverse transcriptase and 5x miScript HiFlex Buffer, in accordance with the manufacturer's instructions. The reaction was incubated at 37°C for 60 min followed by 95°C for 5 min. Quantitative RT-PCR was performed using miScript SYBR Green PCR Kit (Qiagen, USA). The total volume of the reaction mixture was 6 µL and it contained 2x Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA), 10x miScript Universal Primer, nuclease-free water, 0.2 ng of cDNA and 1 μL of specific forward primer which was designed based on canine mature miRNA sequences and according to mirBase database (http://www.mirbase.org, Supplementary Table 1). Amplifications were performed

using QuantStudio 6 Flex (Thermo Fisher Scientific, USA). The reactions were exposed to 95°C for 15 min, followed by 45 cycles for 15 sec at 94°C, 30 sec at 55°C and 30 sec at 70°C. This was followed by melting curve according to the manufacturer's instructions. We considered the miRNA present when cycle threshold (CT) was less than 37 cycles in at least three biological repetitions with adequate melting curves; CT was normalized using the geometric means of miR-99b, Hm/Ms/Rt T1 sRNA and RNU43snoRNA according to Silveira and colleagues(DA SILVEIRA et al., 2017). Data analyses were performed to evaluate the miRNAs that were described as common, exclusive as well as differently abundant in the Complete Remission and Disease Progression. The analysis of the Oncomirs was carried out in three stages, aiming answer the questions: how many miRNAs were expressed by each group? Were there exclusive miRNAs in the groups? Was there any miRNA that was differently expressed between the groups? First step was the frequency analysis, and the miRNA was considered positive in the group when we had his expression in at least one sample. Second was to investigate exclusive Oncomirs related to CR and/or DP using statistical test of frequency, and third was analyze the difference in Oncomirs expression between CR and DP ($P < 0.1$).

2.3.9. Statistical Analysis

All data were evaluated for homoscedasticity with D'Agostino & Pearson normality test and parametrical or non-parametrical tests were chosen accordingly. For two group comparisons unpaired T test or Mann-Whitney were used; contingency tables were analyzed with Fisher's exact test; survival curves were analyzed with Log-rank (Mantel-Cox) test; ROC curves were used for the predictive value; and multiple linear regression was used for multivariate analysis. P-values were considered significant when <0.05 otherwise stated.

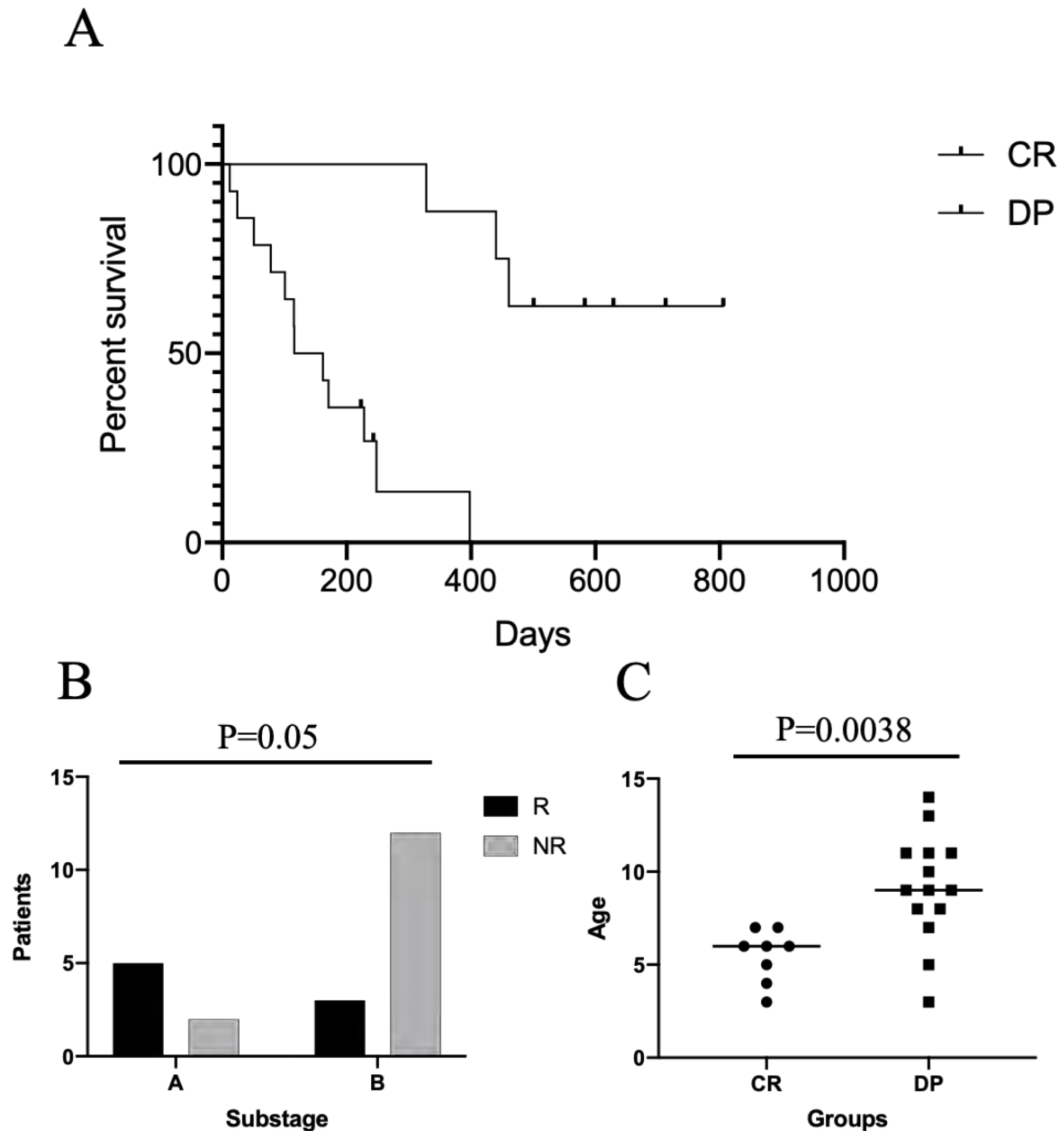
2.4. RESULTS

2.4.1. Patient and control groups

A total of 25 dogs with multicentric lymphadenopathy were cytologically diagnosed with lymphoma and other 30 healthy dogs were used as controls. Age average was 2.43 years (range: 0.5 – 7 years) and 7.8 years (range: 3 – 14 years) in control and lymphoma group respectively. A significant age difference was found between groups (P < 0.0001) and other relevant characteristics from both groups can be found in supplementary table 2. All the 25 cases had the diagnosis of large-cell lymphoma by cytology(PONCE et al., 2010). Only 8 dogs had additional information about diagnosis (immunohistochemistry and/or PARR) being 6 dogs diagnosed as DLBCL by immunohistochemistry(VALLI et al., 2011) and 2 diagnosed as B-cell lymphoma by clonality test PARR(LANGNER et al., 2014) (supplementary table 3).

All patients were treated with the standard CHOP protocol. Evaluation of therapeutic response showed that 8/25 dogs achieved complete remission, 14/25 did not respond to chemotherapy and had disease progression and 3 patients were censored because discontinued the chemotherapy. Overall survival was significantly different between CR and DP groups (P = 0.0002, Fig. 1) with an average survival of 155 days and 553 days, respectively.

Figure 1. Therapeutic response of lymphoma patients and clinical differences between the group of complete remission and the group of disease in progression.



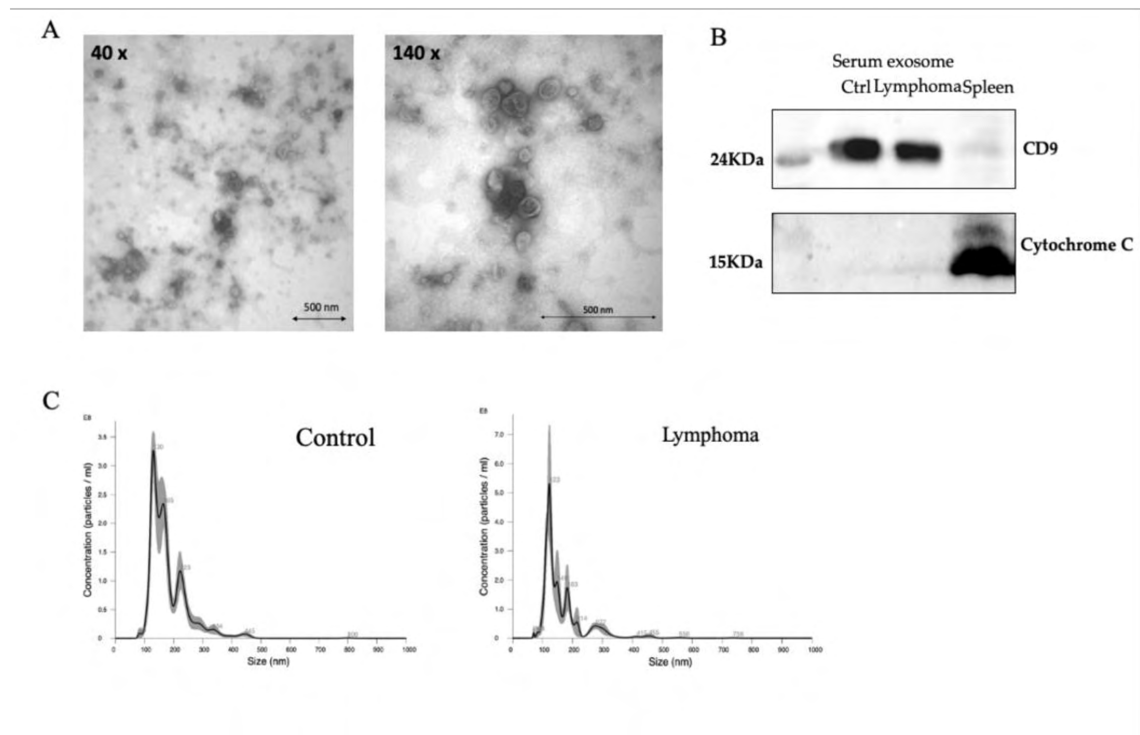
Source: Garnica, T. K. (2020).

In addition, there were differences between CR and DP groups for sub-stage (P=0.05, Fig. 1) and age (P=0.0038, Fig. 1), where the DP patients presented significantly more systemic signs and are older than CR patients.

2.4.2. Characterization of the exosomes isolated from canine serum

Exosomes isolated from the serum of control and lymphoma patients had a typical “donut-like” appearance by transmission electron microscopy (Fig. 2). The NTA analysis showed a range of diameter between 30 – 150 nm agreeing to literature (THIERRY et al., 2006). Exosomes from representative cases (1 case from lymphoma group and 1 from control group) were positive for CD9 and negative for Cytochrome C (Fig. 2). No difference was found on concentration and size of exosomes in sera from lymphoma and control groups.

Figure 2. Characterization of exosomes isolated from canine serum. (A) Transmission electron microscopy showing exosomes with lipid bilayer (Scale bar 500nm). (B) Western blotting analysis of proteins CD9 and Cytochrome C in exosomes and canine tissue (Spleen). The CD9, a transmembrane protein from tetraspanin family, were detected in exosomes from canine serum (control and lymphoma) and tissue (spleen). The Cytochrome C, a mitochondrial marker, was only detected in spleen, thus confirming the absence of cell contamination in exosome samples. (C) The NTA analysis of the particle size (nm) of exosomes isolated from control and lymphoma group showed particles around 30 - 150 nm in diameter.

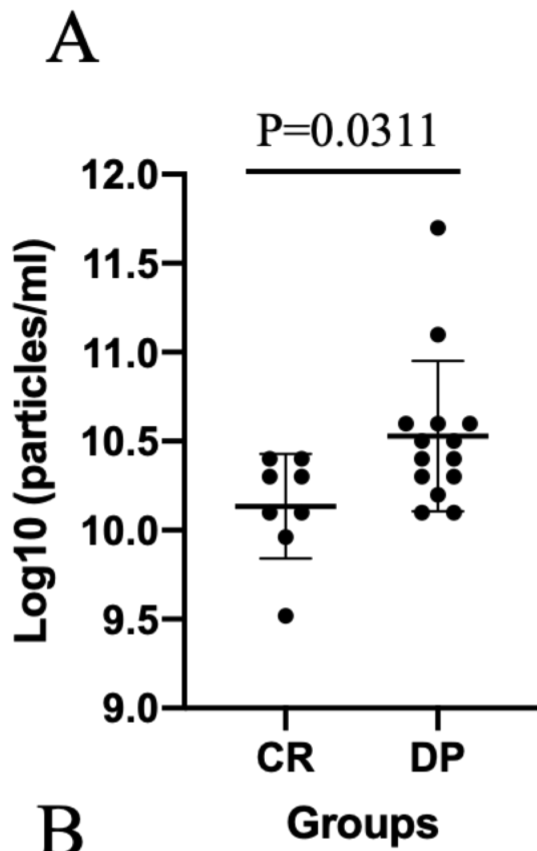


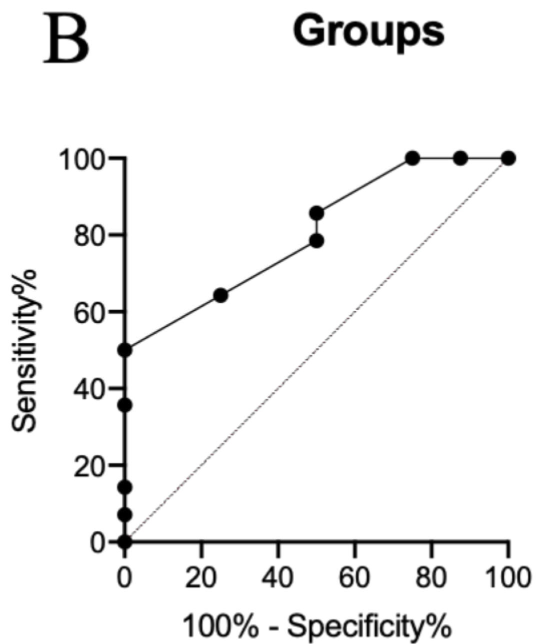
Source: Garnica, T. K. (2020).

2.4.3. The concentration of blood exosomes at diagnosis predicted the response to chemotherapy and Outcome

Twenty-two dogs (8 CR and 14 DP) were evaluated to test the predictive and prognostic value of exosomes size and concentration. Patients with disease progression had higher concentration of serum exosomes at the moment of diagnosis (D0, P = 0.311, Fig. 3). The ROC curve analysis was significant and the Area Under the Curve (AUC) for exosome concentration to predict the response to CHOP was 0.8036 (P = 0.0203, Fig. 3). No association of the size of exosomes was found as predictive or prognostic for lymphoma patients.

Figure 3. Serum exosomes at the diagnosis predicted the response to chemotherapy.





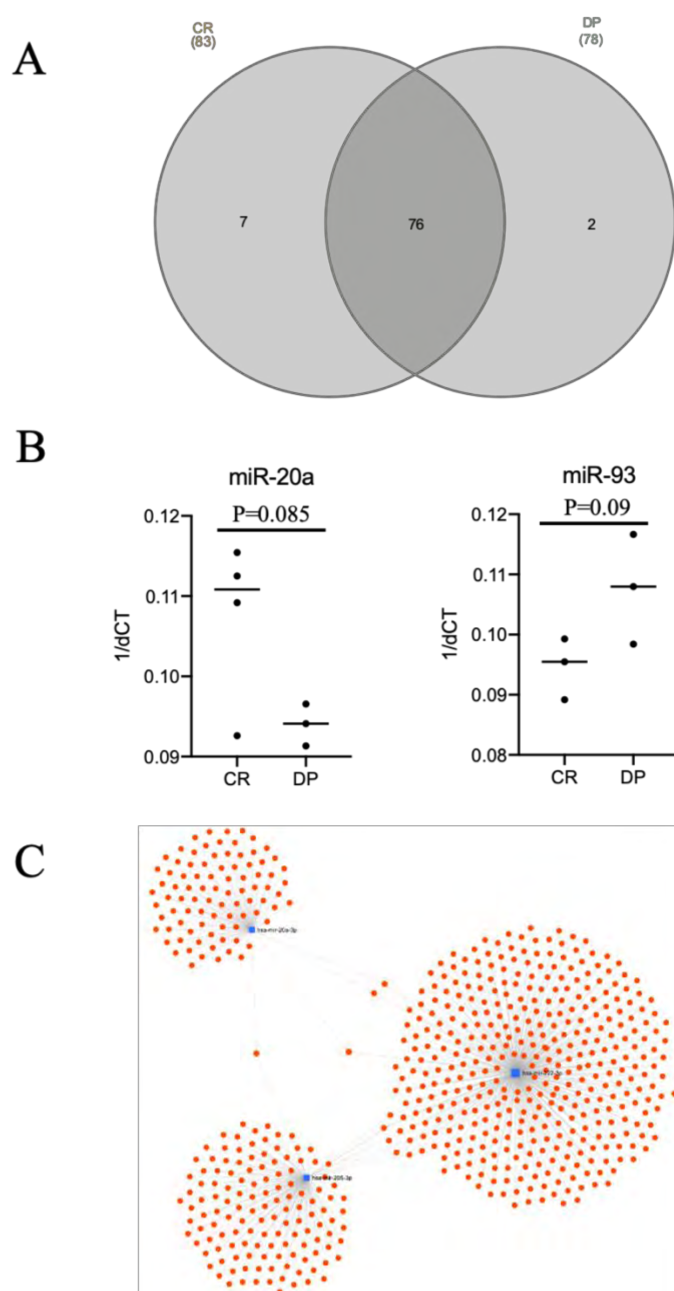
Source: Garnica, T. K. (2020).

Two multiple linear regressions were calculated to predict therapeutic response based on the significant parameters associated with disease progression: the first using age and sub-stage and the second adding exosome concentration. Both regression equations were found highly significant ($F(2,20) = 20.45$, $P<0.0001$) and ($F(3,19) = 15.68$, $P<0.0001$), respectively. When exosome concentration was included in the model the R_2 increased from 0.69 to 0.73, suggesting an improvement in the prediction of therapeutic response.

2.4.4. Oncomirs screening revealed potential markers for clinical response

The screening of 95 oncomirs in representative exosome samples (5 from CR and 5 from DP groups) showed that 85 oncomirs were found in at least one patient (Fig. 4). In addition, 76 oncomirs were found in both groups, 7 were found only in the CR group (miR-151-5p, miR-190a, miR-200c, miR-204, miR-488, miR-183, miR-205) and 2 were found only in DP group (miR-196a, miR-10b, Fig. 4). Statistical analysis showed 2 oncomirs with higher frequency in the CR group: miR-205 (P= 0.0384, Fig. 4) and miR-222 (P=0.0578, Fig. 4). When we compared the expression level, 2 oncomirs were differentially expressed between CR and DP groups. The mir-20a was more expressed in patients with CR (P = 0.085, Fig. 4), while miR-93 was more expressed in patients with DP (P = 0.09, Fig. 4). The pathway analysis performed for oncomirs found in the CR group (miR-20a, miR-205 and miR-222) enriched for pathways involved in activation of BH3-only proteins (P = 0.009), PIP3 activating Akt signaling (P = 0.018) and signaling by SCF-kit (P = 0.018, table 1).

Figure 4. Screening of exosomal oncomirs revealed potential markers for clinical response. (A) Venn diagram of 85 Oncomirs found to be expressed in exosomes. (B) Expression of mir-20a and mir-93 is significantly different between groups CR and DP. (C) Target genes from the Oncomirs associated with Complete Remission Lymphoma patients. It is possible to notice few genes targeted for more than one oncomir.



Source: www.mirbase.org

Table 1. Enriched pathways for oncomirs in the Complete Remission Group.

Pathway	Expected	Hits	Pval
Gene Expression	32.2	65	1.13e-05
Activation of BH3-only proteins	0.869	7	0.00941
BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	0.302	4	0.018
PI3K events in ERBB4 signaling	3.55	12	0.018
PIP3 activates AKT signaling	3.55	12	0.018
Signaling by SCF-KIT	5.03	15	0.018
PI3K events in ERBB2 signaling	3.55	12	0.018
Cellular responses to stress	9.67	23	0.018
Oncogene Induced Senescence	1.13	7	0.018
Role of LAT2/NTAL/LAB on calcium mobilization	3.89	13	0.018
PI-3K cascade:FGFR2	3.55	12	0.018
PI-3K cascade:FGFR3	3.55	12	0.018
PI-3K cascade:FGFR4	3.55	12	0.018
PI-3K cascade:FGFR5	3.55	12	0.018
PI3K/AKT activation	3.66	12	0.0227
GAB1 signalosome	3.7	12	0.0227
Cellular Senescence	5.4	15	0.0227
Intrinsic Pathway for Apoptosis	1.36	7	0.0234
Cell Cycle	18.8	34	0.0325

Source: www.mirbase.org

2.5. DISCUSSION

The chemoresistance and relapse are events commonly observed during the treatment of canine lymphomas being directly related to therapeutic efficacy and survival(THAMM, 2019). Data from the literature show that 85% of dogs with lymphoma treated with CHOP 19 weeks protocol, achieves complete or partial response(VAIL; PINKERTON; YOUNG, 2013). However, approximately 50% of dogs relapse the disease within 1 year after diagnosis(MARCONATO et al., 2016; ZANDVLIET, 2016b; THAMM, 2019). The relapse decreases the chances of achieve remission again and respond to secondary protocols(BURTON; GARRETT-MAYER; THAMM, 2013). Thus, the ability to predict the cases that will not respond to CHOP are highly needed. In this work, we show that a liquid biopsy based on exosomes of patients with multicentric lymphoma can predict the response to CHOP protocol and the patients who will die due to lymphoma with an accuracy of 76% and 83%, respectively. Also, we found exosomal oncomirs associated with response to CHOP protocol that can be used to improve the ability of the liquid biopsy in the future.

In our study, lymphoma patients that didn’t respond to chemotherapy have disease progression and significant short overall survival. We found increased concentration of serum exosomes at the diagnosis in these patients when comparing to patients that have complete remission. However, there was no difference between lymphoma patients and control patients regarding exosome concentration. At a first glance, one may consider this a setback, but we shall not forget that the major need nowadays is to predict response to chemotherapy and survival and not to diagnoses lymphoma which can be done easily by the clinician. In addition, evaluation of therapeutic response based only in clinical signs and lymph node measurement can result in misdiagnosis of complete remission(MARCONATO et al., 2016; SATO et al., 2016; CUNNINGHAM; IYENGAR; SHARMA, 2017; PARISSENTI et al., 2019), corroborating the need of more quantitative markers to help evaluate therapeutic response and improve lymphoma treatment.

We also found that age at the diagnosis and the presence of systemic symptoms (sub-stage B) are related to shorter overall survival and both are significant for prediction of therapeutic response. The substage B is a well-known prognostic factor for response to chemotherapy of canine lymphomas (KELLER et al., 1993; GARRETT et al., 2002). No other clinical data was associated with therapeutic response, and since we didn't have immunophenotyping or histopathological analysis for all patients we didn't consider their possible association with therapeutic response. A study conducted by Moore and colleagues found that anemia and high body weight in elderly dogs associated to poor prognosis in lymphoma patients(MOORE; FRIMBERGER, 2018), corroborating the data in our study since 13 out 14 dogs from DP group have systemic signs (sub-stage B). Interestingly, the addition of the exosome concentration analysis in the multiple linear regression model increased ~10% the R_2 improving the model to predict therapeutic response. In humans, the quantification of blood exosome is a potential prognostic marker for esophageal squamous cell carcinoma (MATSUMOTO et al., 2016) and patients with high levels of exosomes in plasma tended to have shorter overall survival than patients with low levels, corroborating our results(SILVA et al., 2012).

Our screening of 95 oncomirs in blood exosomes revealed 4 possible markers related to the outcome of lymphoma patients: mir-205 was found exclusively in the complete remission group; mir-222 have a significant higher frequency in CR group; mir-20a was more expressed in the group CR and only mir-93 was more expressed in the DP group.

Functional enrichment analysis of miR-205, miR-222 and miR-20a in CR group showed involvement in activation of BH3-only proteins (P = 0.009), PIP3 actives AKT signaling (P = 0.018) and signaling by SCF-kit (P = 0.018). The BH-3 only group of pro-apoptotic Bcl-2 proteins consists of Bim (BCL2L11), Puma/BBC3, Bad (Bcl-2/Bcl-x-associated death promoter), Bid (BH-3 interacting-domain death

agonist), Bik (Bcl-2-interacting killer), Noxa/PMAIP1, Bmf (Bcl-2-modifying factor), and Hrk (Harakiri) and are essential for initiating the apoptotic cascade. Loss of BH3-only proteins is involved in B-cell lymphomagenesis(FRENZEL et al., 2010). The PI3K/AKT/mTOR is an important pathway in cancer related with competitive growth, survival, increase metastatic ability and resistance to conventional therapy and also has been explored for canine oncology(CHEN et al., 2012; WESTIN, 2014). The whole genome and exosome sequencing show B-cell and T-cell lymphoma mutated genes related to dysregulation of the PI3k/PTEN signaling axis in canine lymphomas(ZHANG et al., 2013; ELVERS et al., 2015). The inhibitor of PI3Kδ is on phase I/II for canine spontaneous lymphoma as adjuvant therapy for recurrent and refractory cases(GARDNER et al., 2018). SCF-kit is the complex composed by ligand SFC and tyrosine kinase receptor c-Kit. The gene c-Kit is a proto-oncogene that is subject to dysregulation and gain-of-function mutations and amplifications that promote tumorigenesis in a variety of tumor types(MAZZOLDI et al., 2019). However, c-Kit can also promote apoptosis and inhibit tumor growth in absence of SCF (stem cell factor)(WANG et al., 2018).

The miR-205 was exclusively expressed in CR group. This gene can acts as a tumor suppressor or oncogene depending on specific cancer context or its target genes(QIN et al., 2013). There is no information in literature about the expression of miR-205 in dogs with lymphoma, but in humans low expression level of this miRNA is associated with B-cell lymphomas(YAMAGISHI et al., 2015). The miR-222 was higher expressed in CR group and was associated with good prognosis. Contradictory results recently published showed miR-222 expression in plasma of dogs with B-cell or T-cell lymphoma was negatively correlated with OS and PFS(CRAIG et al., 2019).

The miR-20a was more expressed in CR group and were related to a good prognosis. The miR-20a is a member of mir-17-92 cluster and is found expressed in B-cell lymphomas and B-cell chronic lymphocytic leukemia in humans (MOGILYANSKY; RIGOUTSOS, 2013). The plasma levels of miR-20a was associated with high mortality in humans with DLBCL(KHARE et al., 2017). In dogs,

there is one report showing increased miR-20a in plasma of T-cell lymphoma patients in comparison to control animals[34]. At last, we found only the miR-93 being more expressed in DP group. The miR-93 is a precursor of miR-17 family and its expression is associate to aggressive cancer phenotypes due increasing metastasis, tumor growth, invasion and angiogenesis in different human cancer as NSCLC, ovarian cancer, gastric cancer and glioma(FANG et al., 2011; GUAN et al., 2017; LI; LYU; MENG, 2017; LIU et al., 2018). Khare *et al* 2017 showed that miR-93 is downregulated in plasma of humans diagnosed with Hodgkin lymphoma (HL), and it is also associated high mortality rate in DLBCL(KHARE et al., 2017), supporting our results of exosomal increased levels of miR-93 in non-responder's group.

Taken together, these data suggest a possible molecular signature for exosome content based on oncomirs related to the outcome of lymphoma patients, but additional experiments are needed to validate these findings using more samples. In fact, other studies already showed the potential of microRNAs as potential biomarkers in canine cancers (HEISHIMA et al., 2017; CRAIG et al., 2019).

One of the challenges of our study was to find multicentric lymphoma cases that fit in proposed inclusion criteria. Lymphoma has higher incidence in older dogs (>10 years) which increases the chances of concomitant diseases. Other challenge was to find patients with complete information about diagnosis, immunohistochemistry and histopathology. This is explained by the fact that some clinicians consider the therapeutic approach just after the cytologic diagnosis, not recommending the biopsy for further histopathology/immunophenotyping analyses. A study conducted by Regan and colleagues during the 2009 Veterinary Cancer Society Annual Conference interviewed 519 clinicians and showed that 76% of volunteers recommended the immunophenotyping but only 28% recommended the lymph node histopathology for full staging. Probably in our field conditions (Brazil) these numbers are even lower mostly due to few numbers of good diagnostic laboratories across the country and the price of the tests.

To the best of our knowledge, this is the first study to show the potential of predicting response to CHOP and outcome of lymphoma patients using a liquid biopsy based on exosomes and their miRNAs content. There are two studies recently published involving molecular markers for prediction. Parissenti by *et al* 2019 revealed that RNA disruption in affected lymph nodes can be associated with good response to CHOP chemotherapy(PARISSENTI et al., 2019). Other study evaluated the miRNAs concentration in plasma and identify 8 miRNAs had differential expression in the non-remission group compared to dogs that completed CHOP in complete remission(CRAIG et al., 2019). Regardless these findings, the literature about predictive markers for canine multicentric lymphoma continues limited.

In conclusion, the results generated in this work can trigger advances to veterinary oncology by introduction of liquid biopsy approach which can helps the clinicians to early identify refractory patients and help to guide the best treatment possible. The validation of the oncomir's signature of lymphoma patients who didn't respond to chemotherapy will have the potential to guide future studies on targeted therapies opening a window to precision medicine in veterinary oncology.

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Supplementary table 1. Primers for Oncomir screening

Target	Sequence (5'-3')
bfa-miR-122	TGGAGTGTGACAATGGTGTTTG
bta-let-7a-5p	TGAGGTAGTAGGTTGTATAGTT
bta-let-7b	TGAGGTAGTAGGTTGTGTGGTT
bta-let-7c	TGAGGTAGTAGGTTGTATGGTT
bta-let-7d	AGAGGTAGTAGGTTGCATAGTT
bta-let-7e	TGAGGTAGGAGGTTGTATAGT
bta-let-7f	TGAGGTAGTAGATTGTATAGTT
bta-let-7g	TGAGGTAGTAGTTTGTACAGTT
bta-let-7i	TGAGGTAGTAGTTTGTGCTGTT
bta-miR-103	AGCAGCATTGTACAGGGCTATGA
bta-miR-106b	TAAAGTGCTGACAGTGCAGAT
bta-miR-125b	TCCCTGAGACCCTAACTTGTGA
bta-miR-126-5p	CATTATTACTTTTGGTACGCG
bta-miR-128	TCACAGTGAACCGGTCTCTTT
bta-miR-132	TAACAGTCTACAGCCATGGTCG
bta-miR-134	TGTGACTGGTTGACCAGAGTGG
bta-miR-135b	TATGGCTTTTCATTCCTATGTGA
bta-miR-136	ACTCCATTTGTTTTGATGATGGA
bta-miR-145	GTCCAGTTTTCCCAGGAATCCCT
bta-miR-149-5p	TCTGGCTCCGTGTCTTCACTCCC
bta-miR-151-5p	TCGAGGAGCTCACAGTCTAGT
bta-miR-155	TTAATGCTAATCGTGATAGGGGT
bta-miR-15a	TAGCAGCACATAATGGTTTGT
bta-miR-16b	TAGCAGCACGTAAATATTGGC
bta-miR-181d	AACATTCATTGTTGTCGGTGGGT
bta-miR-185	TGGAGAGAAAGGCAGTTCCTGA
bta-miR-186	CAAAGAATTCTCCTTTTGGGCT
bta-miR-188	CATCCCTTGCATGGTGGAGGGT
bta-miR-18a	TAAGGTGCATCTAGTGCAGATA
bta-miR-190a	TGATATGTTTGATATATTAGGT
bta-miR-190b	TGATATGTTTGATATTGGGTT
bta-miR-194	TGTAACAGCAACTCCATGTGGA
bta-miR-195	TAGCAGCACAGAAATATTGGCA
bta-miR-196a	TAGGTAGTTTCATGTTGTTGGG
bta-miR-197	TTCACCACCTTCTCCACCCAGC
bta-miR-200b	TAATACTGCCTGGTAATGATG
bta-miR-200c	TAATACTGCCGGGTAATGATGGA

bta-miR-204	TTCCCTTTGTCATCCTATGCCT
bta-miR-205	TCCTTCATTCCACCGGAGTCTG
bta-miR-206	TGGAATGTAAGGAAGTGTGTGG
bta-miR-20a	TAAAGTGCTTATAGTGCAGGTAG
bta-miR-210	ACTGTGCGTGTGACAGCGGCTGA
bta-miR-214	ACAGCAGGCACAGACAGGCAGT
bta-miR-22-3p	AAGCTGCCAGTTGAAGAACTG
bta-miR-221	AGCTACATTGTCTGCTGGGTTT
bta-miR-222	AGCTACATCTGGCTACTGGGT
bta-miR-24	GTGCCTACTGAGCTGATATCAGT
bta-miR-25	CATTGCACTTGTCTCGGTCTGA
bta-miR-26a	TTCAAGTAATCCAGGATAGGCT
bta-miR-26b	TTCAAGTAATTCAGGATAGGTT
bta-miR-27a-3p	TTCACAGTGGCTAAGTTCCG
bta-miR-27b	TTCACAGTGGCTAAGTTCTGC
bta-miR-29b	TAGCACCATTTGAAATCAGTGTT
bta-miR-29c	TAGCACCATTTGAAATCGGTTA
bta-miR-30b-5p	TGTAAACATCCTACACTCAGCT
bta-miR-30c	TGTAAACATCCTACACTCTCAGC
bta-miR-423-5p	TGAGGGGCAGAGAGCGAGACTTT
bta-miR-488	TTGAAAGGCTGTTTCTTGGTC
bta-miR-92a	TATTGCACTTGTCCCGGCCTGT
bta-miR-92b	TATTGCACTCGTCCCGGCCTCC
bta-miR-93	CAAAGTGCTGTTCGTGCAGGTA
cfa-miR-1-1	TGGAATGTAAAGAAGTATGTA
cfa-miR-101	TACAGTACTGTGATAACTGA
cfa-miR-106a	AAAGTGCTTACAGTGCAGGTAG
cfa-miR-107	AGCAGCATTGTACAGGGCTAT
cfa-miR-10b	CCCTGTAGAACCGAATTTGTGT
cfa-miR-125a	TCCCTGAGACCCTTTAACCTGT
cfa-miR-133a	TTGGTCCCCTTCAACCAGCTGT
cfa-miR-137	TTATTGCTTAAGAATACGCGT
cfa-miR-140	ACCACAGGGTAGAACCACGGA
cfa-miR-141	AACACTGTCTGGTAAAGATGG
cfa-miR-142	CCCATAAAGTAGAAAGCACTA
cfa-miR-143	TGAGATGAAGCACTGTAGCTC
cfa-miR-146a	TGAGAACTGAATTCCATGGGTT
cfa-miR-150	TCTCCCAACCCTTGTACCAGTG
cfa-miR-153	TTGCATAGTCACAAAAGTGA
cfa-miR-15b	TAGCAGCACATCATGGTTTA
cfa-miR-181a	AACATTCAACGCTGTCGGTGAG

cfa-miR-181b	AACATTCATTGCTGTCGGTG
cfa-miR-181c	AACATTCAACCTGTCGGTGAGTT
cfa-miR-183	TATGGCACTGGTAGAATTCACT
cfa-miR-191	CAACGGAATCCCAAAAGCAGCT
cfa-miR-192	CTGACCTATGAATTGACAGCC
cfa-mir-199	ACAGTAGTCTGCACATTGGTT
cfa-miR-200a	CATCTTACCGGACAGTGCTGGA
cfa-miR-202	TTCCTATGCATATACTTCTTTG
cfa-miR-21	TAGCTTATCAGACTGATGTTGA
cfa-miR-215	ATGACCTACGAATTGATAGACA
cfa-miR-218	TTGTGCTTGATCTAACCATGT
cfa-miR-223	TGTCAGTTTGTCAAATACCCC
cfa-miR-224	CAAGTCACTAGTGGTTCCGTTT
cfa-miR-23a	ATCACATTGCCAGGGATTT
cfa-miR-29a	TAGCACCATCTGAAATCGGTTA
cfa-miR-30a	TGTAAACATCCTCGACTGGAAGC
cfa-miR-9	TCTTTGGTTATCTAGCTGTATGA
bta-miR-99b (Housekeeping_1)	CACCCGTAGAACCGACCTTGCG
Hm/Ms/Rt T1 snRNA (Housekeeping_2)	CGACTGCATAATTTGTGGTAGTGG
RNT43 snoRNA (Housekeeping_3)	CTTATTGACGGGCGGACAGAAAC

Supplementary table 2. Statistical analysis of data comparing control group and lymphoma patients.

	Lymphoma group (n = 25)	Control Group (n = 30)	P value
Sex	Male n = 14 Female n = 11	Male intact n = 14 Female n = 16	0.591
Age	Male 3 – 13 years (mean 7.8) Female 3 – 14 years (mean 7.7)	Male 1 – 15 years (mean 4.1) Female 0.5 – 13 years (mean 2.7)	<0.0001
Breed	Mixed Breed n = 9 Lhasa Apso n = 2 Labrador Retriever n = 2 Golden Retriever n = 1 Boxer n = 2 Yorkshire n = 1 West High England terrier n = 1 Pit Bull n = 1 Doberman Pinscher n = 1 Poodle n = 1 Dachshund n = 1 French Bulldog n = 1 American Bully n = 1 White Swiss Shepperd Dog n = 1	Mixed Breed n = 13 Border Collie n = 3 Shi Tzu n = 2 Labrador Retriever n = 2 Golden Retriever n = 2 Australian Cattle dog n = 1 Lhasa Apso n = 1 Maltese n = 1 Yorkshire n = 1 Pit Bull n = 1 Doberman Pinscher n = 1 White Swiss Shepperd Dog n = 1 German Shepperd dog n = 1	0.1091

Source: Garnica, T. K. (2020).

Supplementary table 3. Data of lymphoma patients engaged in the study.

Sample	**Hospital**	**Sex**	**Age (years)**	**Breed**	**Weight (Kg)**	**Cytology**	**IMH**	**PARR**	**Stage**	**Substage**	**CHOP (weeks)**	**Therapeutic Response**	**Outcome**	**Relapse**	**OS**	**DFS**
CENSORED_1	FCAV	M	8,0	Mixed Breed	17,6	Large-cell lymphoma			4	B	4	NI	NI		NI	NI
CENSORED_2	FCAV	M	6,0	Mixed Breed	32	Large-cell lymphoma	DLBCL		4	B	4	NI	NI		NI	NI
CENSORED_4	UAM	F	8,0	Dachshund	8	Large-cell lymphoma			4	B	NI	NI	NI		NI	NI
NR_1	FCAV	F	8,0	Mixed Breed	32,5	Large-cell lymphoma			5	B	4	DP	Dead		24,00	
NR_10	UAM	F	10,0	Mixed Breed	33,35	Large-cell lymphoma			NI	NI	3	DP	Dead		116,00	
NR_11	UAM	F	11,0	West High England Terrier	9	Large-cell lymphoma			5	A	4	DP	Dead	yes	171,00	
NR_12	UAM	M	13,0	Yorkshire Terrier	9	Large-cell lymphoma			4	B	19	DP	NI	yes	223,00	35,00
NR_13	UAM	M	5,0	Lhasa Apso	5,7	Large-cell lymphoma			5	B	4	DP	Dead		101,00	
NR_15	UAM	M	3,0	French Bulldog	9,3	Large-cell lymphoma			4	A	19	DP	Dead	yes	398,00	119,00
NR_2	FCAV	M	9,0	Mixed Breed	12,5	Large-cell lymphoma			4	B	4	DP	Dead	yes	51,00	
NR_3	FCAV	M	9,0	Boxer	20	Large-cell lymphoma			5	B	1	DP	Dead		12,00	
NR_4	FCAV	M	11,0	Mixed Breed	9,3	Large-cell lymphoma			4	B	4	DP	Dead		78,00	
NR_5	FCAV	M	9,0	White Swiss Shepperd Dog	47	Large-cell lymphoma			5	B	8	DP	Dead	yes	115,00	
NR_6	FCAV	F	7,0	Pitbull	21,6	Large-cell lymphoma			4	B	7	DP	Dead	yes	228,00	
NR_7	UAM	M	11,0	Pinscher	3,15	Large-cell lymphoma			4	B	12	DP	Dead		162,00	

NR_8	UAM	F	14,0	Poodle	8,1	Large-cell lymphoma	DLBCL		4	B	19	DP	Dead		248,00	
NR_9	UAM	M	8,0	Boxer	30,3	Large-cell lymphoma			4	B	19	DP	NI	yes	243,00	17,00
R_1	FCAV	M	4,0	Labrador Retriever	38,3	Large-cell lymphoma		B-Cell	5	B	19	RC	Alive		713,00	524,00
R_2	FCAV	F	5,0	Mixed Breed	8,9	Large-cell lymphoma	DLBCL		3	A	19	RC	Dead		440,00	286,00
R_3	FCAV	F	6,0	Golden Retriever	45,5	Large-cell lymphoma	DLBCL		3	A	19	RC	Alive	yes	583,00	248,00
R_4	FCAV	F	3,0	American Bully	26,5	Large-cell lymphoma		B-Cell	4	B	19	RC	Dead	yes	328,00	90,00
R_5	FCAV	F	6,0	Mixed Breed	18,5	Large-cell lymphoma	DLBCL		3	A	19	RC	Alive		501,00	
R_6	FCAV	M	6,0	Lhasa Apso	6,5	Large-cell lymphoma	DLBCL		4	A	19	RC	Dead	yes	461,00	314,00
R_7	FCAV	F	7,0	Mixed Breed	20,9	Large-cell lymphoma			3	A	19	RC	Alive		806,00	638,00
R_8	UAM	M	7,0	Labrador Retriever	45,5	Large-cell lymphoma			4	B	19	RC	Alive		629,00	

Legend: Cytology according to Ponce et al 2010; Immunohistochemistry according to Langner et al 2014; PARR according to Valli et al 2011, NI = no information available.

Source: Garnica, T. K. (2020).

3. ARTICLE 2:

INVESTIGATING THE ROLE OF EXOSOMES DERIVED FROM CHEMOTHERAPY RESISTANT LYMPHOMA CELLS AS MEDIATORS OF CELLULAR PLASTICITY

3.1. ABSTRACT

The Non-Hodgkin lymphoma (NHL) is a type of cancer that occurs in humans and dogs, it is a heterogenous disease with than 60 subtypes in humans and 38 different subtypes in dogs. Standard treatment consists of chemotherapy but the recurrence and the development of chemoresistance are common for both species. Currently, there is a lack of knowledge of the molecular mechanisms underpinning the development of chemoresistance in lymphomas. Exosomes are small extracellular vesicles (30-150 nm in diameter) involved in intercellular communication and biological material transference (including mRNA, miRNA, lipids, and metabolites) between the cells. Exosomes are an emerging key to understand cancer cell communication and signaling pathway activation during the chemoresistance process. Our hypothesis is that exosomes secreted by chemo-resistant (CR) lymphoma cells can drive phenotypic changes in non-resistant lymphoma cells. For this purpose, we induced chemoresistance using the CHOP protocol (dexamethasone, doxorubicin and vincristine cocktail) in 3132 cells (canine lymphoma B cell) and Jurkat cells (human T cell leukemia). We showed that Jurkat cells are more tolerant to dexamethasone comparing to 3132 cells, and determined IC50 values as 1 nM of dexamethasone, 0.05 μM of doxorubicin and 1 nM of vincristine for Jurkat cells and 3132 cells. Acquisition of chemoresistance was demonstrated by increased cell viability after treatment using IC50 of drugs cocktail. We also show that the 3132-CR and Jurkat-CR have slower doubling time compared to respective naïve cells. Exosomes derived from the putative chemo-resistant and naïve cells, and we show that the 3132 (naïve and CR) released more exosomes after chemotherapy treatment (P=0.0187), but there was no difference in the amount of exosomes secreted between chemo-resistant and naïve cells

(P=0.7661). No significant difference in exosomes production were found in Jurkat cells after chemotherapy. Exosomes derived from the putative chemo-resistant were isolated and used to treat their respective naïve cells. There was an increase in cells proliferation after the exosome's treatment comparing to control for 3132 (P<0.001) and Jurkat (P<0.0035). However, there was no difference in proliferation comparing the treatment using exosomes derived from naïve cells and those derived from CR cells for 3132 (P=0.11) and Jurkat (P=0.91). The cell proliferation was higher after 78 hours of exosome's treatment for 3132 (P<0.001) and Jurkat (P<0.001). These results show that acquisition of chemoresistance induces some phenotypic changes in lymphoma and leukemia cells and that there are changes in the amount of exosomes secreted during the chemotherapy treatment and the development of chemoresistance. However, further experiments are required to prove our initial hypothesis.

Keywords: lymphoma, chemoresistance, exosomes.

3.2. INTRODUCTION

Lymphoma is one of the most frequent cancer in dogs. According to epidemiological data from Brazil, lymphoma is among the three most frequent canine cancers (De Nardi et al. 2004), corresponding to about 80% of neoplasias of hematopoietic origin (Schmidt et al. 2013). The estimated incidence of the disease is 13 to 114 new cases per 100,000 dogs per year (Dobson et al. 2002; Teske 1994). This cancer is characterized by clonal proliferation of malignant lymphocytes originating in lymphoid organs such as bone marrow, thymus, spleen, liver and lymph nodes. However, other tissues may also be affected by the migration and installation of these lymphocytes through the bloodstream (Vail, David M and Young 2013). The non-Hodgkin type (NH) is more diagnosed, corresponding to 90% of the cases. Among the NHL lymphomas, those of B-cell origin are the most common and present a less aggressive behaviour and a more favorable prognosis in relation to multicentric T-cell lymphomas, which represent

about 10-38% of these neoplasms (Rebhun et al. 2011). Diffuse large B-cell lymphoma is the most common histological subtype in the canine population (Valli et al. 2011).

The treatment for canine lymphoma is chemotherapy and the UW-19 protocol (CHOP) is the first therapeutic choice for the treatment of NH lymphomas of any immunophenotype (B and T cells) in dogs (Rebhun et al. 2011). This protocol achieves a complete remission rate of 85% after treatment and treated dogs achieve an average survival of 8 to 12 months. However, CHOP does not have a curative effect, which results in the relapse and death of about half the dogs treated in the period up to one year after the diagnosis and only a quarter of the patients remain in remission for up to two years (Burton, Garrett-Mayer, and Thamm 2013). These data show that there are distinct therapeutic responses between patients with the same diagnosis and that relapse is an important factor directly related to death by the disease.

The high rate of post-treatment relapse can be explained by the induction of chemoresistance mechanisms in neoplastic cells, such as the expression of multidrug chemoreceptor-related genes, such as multi-drug resistance (MDR) (Lee et al. 1996). Another factor related to relapse is the method of diagnosis of remission. The complete remission status is based on the disappearance of the clinical signs and there are no accurate methods and molecular markers that can predict therapeutic response.

Exosomes are vesicles generated by multivesicular bodies derived from cells ranging from 30 nm to 150 nm in diameter and their most important physiological functions are cell communication and macromolecule transmission between cells (Minciacchi, Freeman, and Di Vizio 2015). Exosomes has demonstrated pro-tumorigenic properties, that is, they may play an important role in tumour progression and modulation of cell mechanisms associated with hallmarks (Sharma, Khatun, and Shiras 2016; Yang and Robbins 2011). These vesicles can carry important information for intercellular communication, such as molecules of mRNAs, proteins, lipids and miRNAs related to the development and progression of cancer cells (Sharma et al., 2016). Studies has been shown that exosomes *in vitro* can play a role in tumour heterogeneity transferring metastatic phenotype to a

non-metastatic cell line contributing to accelerate tumor progression affecting cell plasticity and behavior (Schillaci et al. 2017). The exosome transfer of miR-155 demonstrated an important role in induced chemoresistance in breast and pancreatic cancer cells (Patel et al. 2017; Santos et al. 2018).

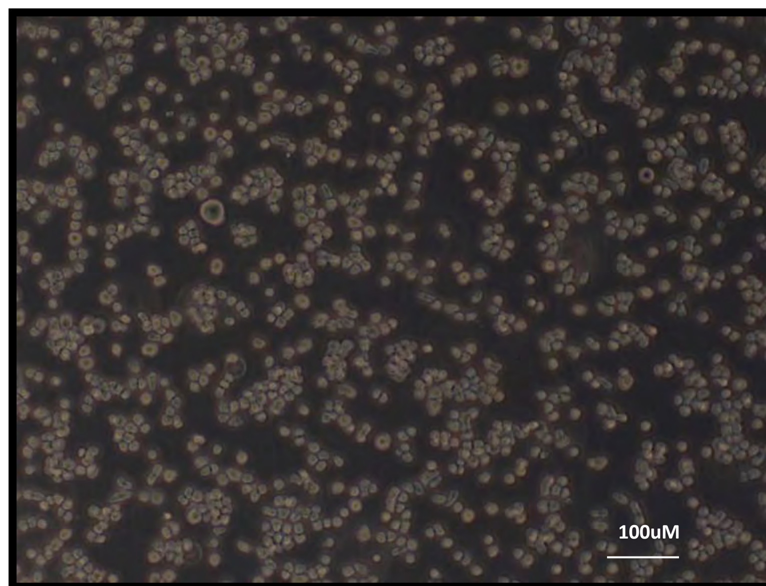
In summary, exosomes can provide a rich source of material to study and better comprehension about chemoresistance and their genetics mechanisms bringing the possibility to discovery and establish new therapeutic targets for lymphoma treatment in both humans and dogs. In additional, there is not many studies published in this field in veterinary oncology. Thereby, our propose is to investigate if chemoresistant lymphoma cells secrete exosomes that can drive phenotypic switching of cancer cell populations and enhance chemoresistant phenotype.

3.3. MATERIAL AND METHODS

3.3.1. Cell culture

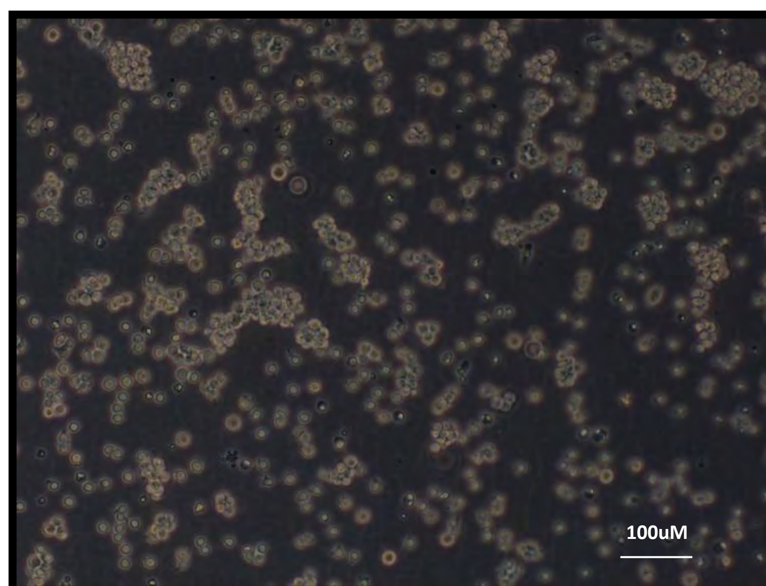
The 3132 (canine B cell lymphoma) and Jurkat clone E6-1 (human T cell leukemia) were provided by Roslin Institute. The cells grew in suspension and were maintained in RPMI 1640 media supplemented with 10% FBS and 1% Pen/Strep.

Figure 1. 3132 (canine B cell lymphoma). High density. 10x magnification.



Source: Garnica, T.K. (2020).

Figure 2. Jurkat (human T cell leukemia). High density. 10x magnification.



Source: Garnica, T. K. (2020).

3.3.2. Doubling-time

To assess the double time for naïve and chemo-resistant cell lines the 3132 naïve, 3132-CR, Jurkat naïve and Jurkat-CR were grown in T75 flasks. The cells were counted every day at the same time for 5 days (0, 24, 48, 72 and 96 hours). This experiment was performed in replicate. The calculation was performed using software online "Doubling Time Online Calculator" available on (http://www.doubling-time.com/compute.php).

3.3.3. Cell cycle assay

The cell cycle assay was performed using Propidium iodide (Invitrogen®) according to protocol for suspension cells. The 2 × 10ˆ5 to 1 × 10ˆ6 cells were centrifugated 1500 rpm, 5 minutes at 4°C. The pellet was resuspended and fixed in cold 70% ethanol and kept -20°C for 30 minutes. The cells were spinned and washed 2 X in PBS. The pellet was treated adding 50ul of RNase (100 µg/ml) and incubated for 90 minutes. At end 200 µl PI (from 50 µg/ml stock solution) were added and the samples, incubated for 30 minutes and evaluated by Flow Cytometry (BD LSR Fortessa™X-20) using Bioimaging and flow cytometry facility at Roslin Institute. This experiment was made in replicate.

3.3.4. IC50 of Doxorubicin, Vincristine and Dexamethasone

Before starting the chemotherapy treatment 3132 and Jurkat naïve cells were treated with different and increasing concentrations for each drug for evaluate the IC50. The cells were seeded in 96 wells plates, cultured for 24 hours and the cell viability was performed following CellTiter-Glo® protocol. The VICTOR3™ Multilabel Counter was used to record the luminescence.

3.3.4.1. Drugs cocktail

To evaluate the maximum tolerated cocktail dose, we prepared 11 cocktails using increasing doses based on IC50 results for each drug. The 3132 and Jurkat naïve cells were plated in 96 wells plate (5x10ˆ2 cells/well) incubated for 24 hours and treated in triplicate using the cocktails. After 72 hours the cell viability was assessed using CellTiter-Glo® and the luminescence was recorded using VICTOR3™ Multilabel Counter.

3.3.5. *Generation of chemotherapy-resistant cell lines*

The protocol was based in previous study published (HARTLEY et al., 2019). Chemotherapy-resistant cell lines were generated by culturing tumour cells in the presence of a mixture of three of the four cytotoxic chemotherapy drugs (doxorubicin, vincristine, and dexamethasone), which comprise the CHOP cocktail, with the exception of cyclophosphamide. For calculate the doses of cocktails we based on the results of IC50 for each drug. We started adding a low dose and see how well the cells survive/die and then add increasing concentrations of the drugs over time (depending on how they grew the dose was increased weekly). The initial doses were: 1/5000th, 1/1000th, 1/100th, 1/10th of the IC50 of all three drugs. The cell lines were grown at the same time using T25 flasks, 4 flasks for each cell according to cocktail dose. After treatment the cell viability was checked every day using Trypan Blue. When the flasks recovered from the last dose (80% of cells alive) they received the treatment again with double dose of cocktail. The cell lines were treated for 10 passages (60 days). We chose the flasks that received the higher doses flask 3 for 3132 and flask 4 for Jurkat as potential chemo-resistant cell lines.

3.3.6. Assessment of chemo-resistant potential

The chemo-resistant phenotype was assessed by cell viability assay using IC50 cocktail dose (1 nM of Dexamethasone, 0.05 μM of Doxorubicin, 1 nM of Vincristine). The same cocktail was used for 3132 and Jurkat. The cells (3132 naïve/CR and Jurkat naïve/CR) were seeded in 6 wells plate 3 x 10ˆ5 cells, treated with cocktail and the cell viability was assessed after 24, 48 and 72-hours using Trypan Blue (Invitrogen®). The experiment was made in triplicates.

3.3.7. Exosome Isolation

The 3132 and Jurkat were grown in exosome free media (exosome depleted serum). The media were harvested when cells acquire 70% of confluency. The media was centrifuged at 1500 rpm, 10 minutes at 4°C. The supernatant was filtered using 0.22 um filter. The samples were spinned at high speed using ultracentrifuge at 120,000g for 70 minutes at 4°C. At the end the pellet was resuspended in 1ml ice cold PBS vortexing 5-7 times to ensure agitation of exosomes from the sides of the tube and frozen at -70°C for further use.

3.3.8. NTA Analysis

The samples were evaluated using Nanosight LM14C (Malvern®, UK) and software NTA 2.3. The samples were diluted 1:10 (range of concentration between 4x10ˆ8 and 12x10ˆ8 particles/ml). The measures were made in duplicate. The higher threshold was 15,000. The samples were recorded for 60 seconds and the lower range for expected particle size was 30nm.

3.3.9. Exosomes release after chemotherapy

To test the hypothesis of increasing exosome releases after chemotherapy the 3132 and Jurkat (naive and chemo-resistant) cells were grown in T75 flasks maintained in exosome free media for 24 hours until 70% of confluency. Then each

cell line received 3 different treatments: IC50 of all three drugs, 50% of IC50 of all three drugs and negative control (exosome-free media). After 24 hours the media was harvested, and the exosomes were isolated. The assay was performed in triplicate.

3.4. Treatment of Naïve cells using exosomes derived from chemo-resistant cell lines

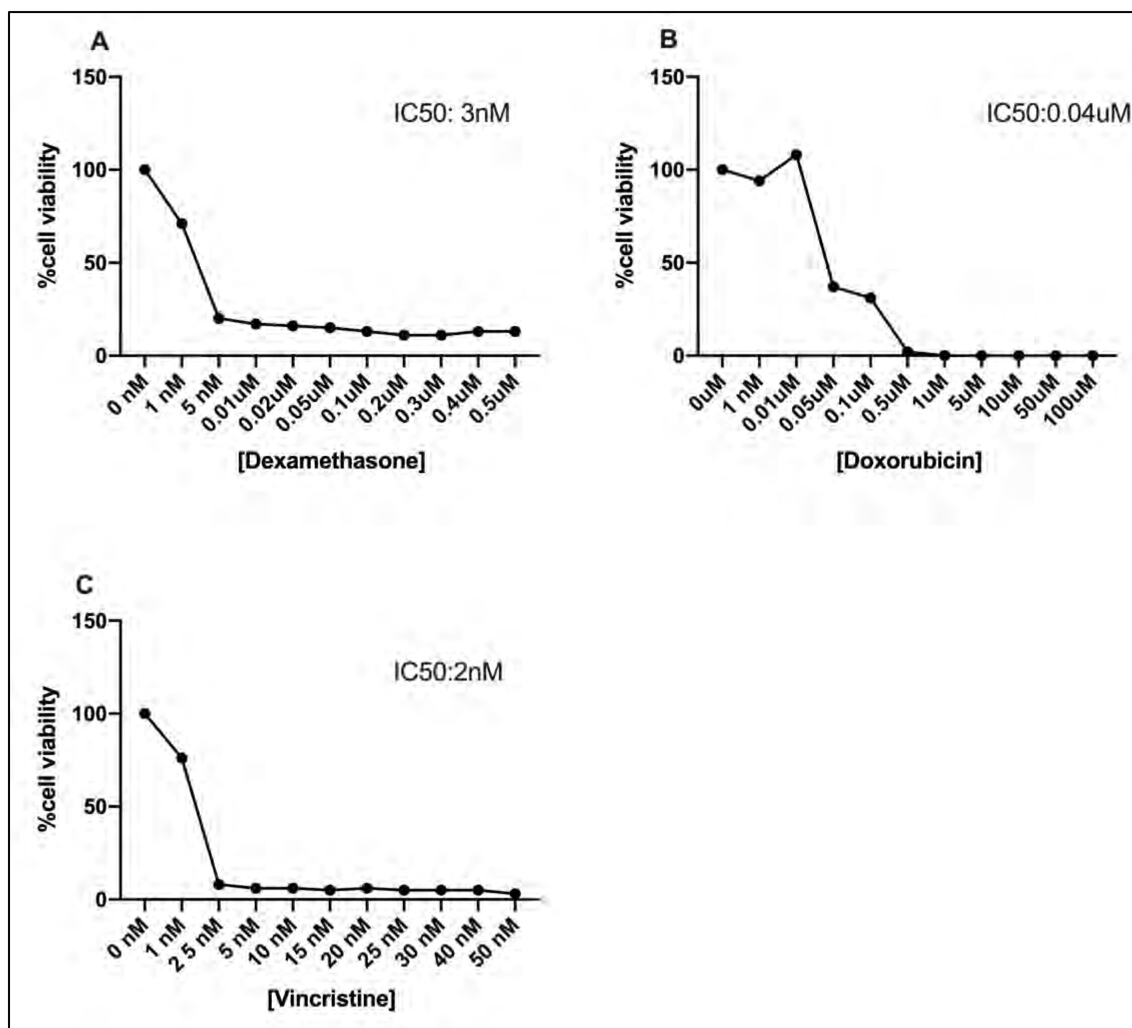
3132 and Jurkat naïve cells were seeded in 96 wells plate (5x10ˆ4 cells/well) and treated using 5 different exosomes titrations (8:1, 4:1, native condition, 1 in 2, 1 in 4). The treatment used exosomes from chemo-resistant and naïve cells lines. Exosome-free media was used as negative control. The increase on cell viability was checked after 48 and 72 hours following the CellTiter-Glo® protocol. The assay was made in duplicate.

3.5. Results and Discussion

3.5.1. IC50 for each drug

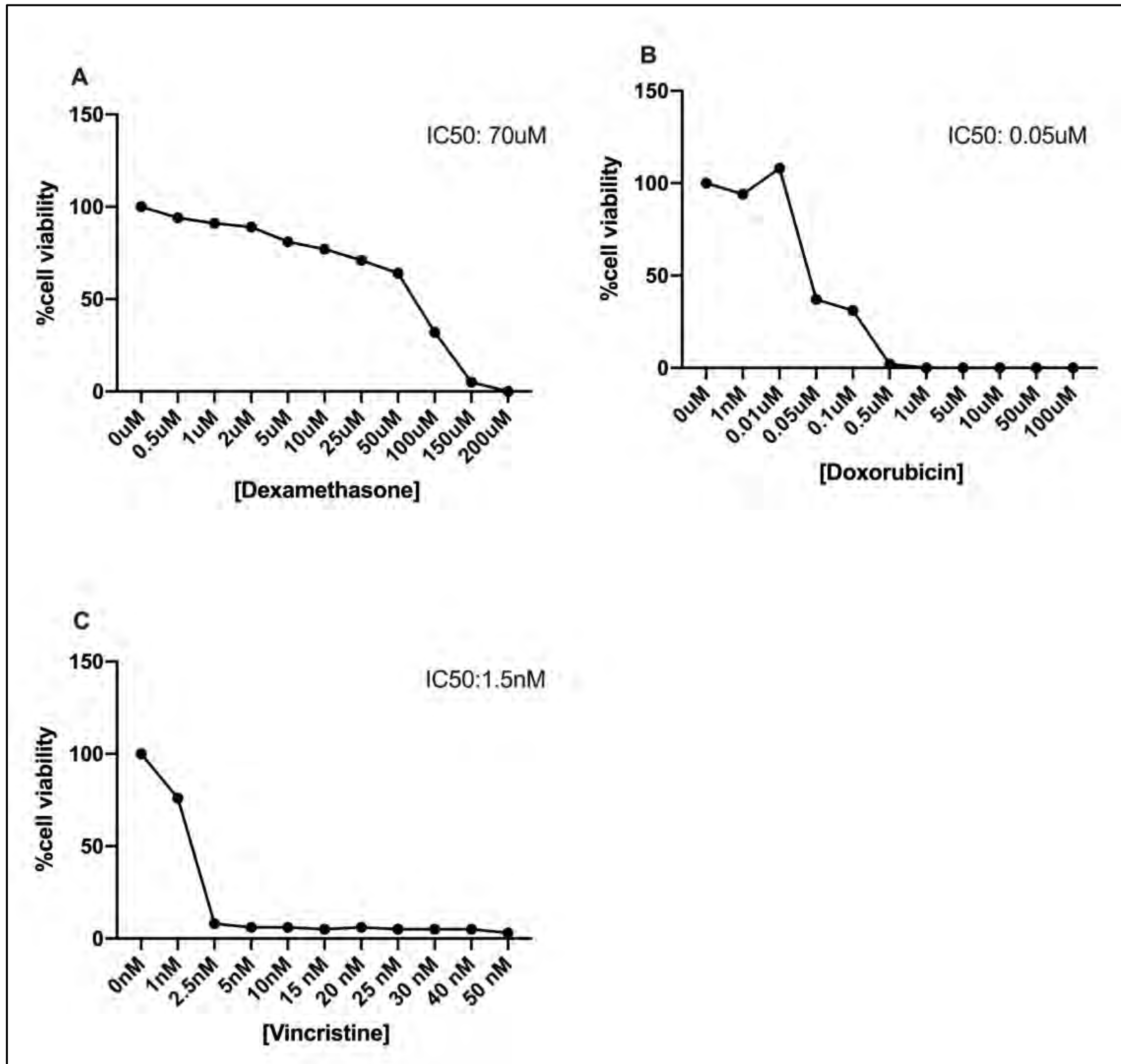
The graphs below show the results about chemosensitivity assay and the respective IC50 for dexamethasone, doxorubicin and vincristine using 3132 (Figure 3) and Jurkat cell lines (Figure 4).

Figure 3. Chemosensitivity Assay for 3132 cell line. A: Dexamethasone, B: Doxorubicin and C: Vincristine.



Source: Garnica, T. K. (2020).

Figure 4. Chemosensitivity Assay for Jurkat cell line. A: Dexamethasone, B: Doxorubicin and C: Vincristine



Source: Garnica, T. K. (2020).

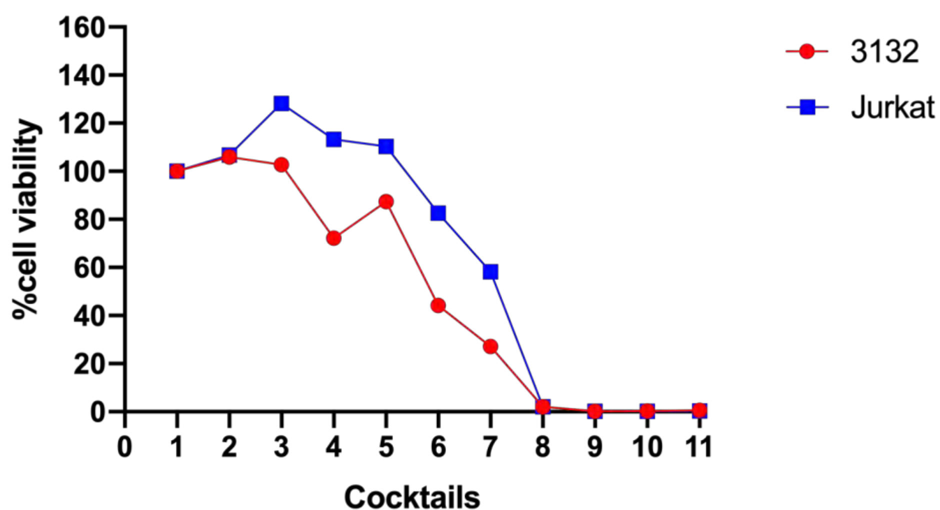
The IC50 for Dexamethasone was quite different between cells. The Dexamethasone IC50 were 3nM and 70 μM for 3132 and Jurkat respectively. This result demonstrated that Jurkat cell is 23.300 times more tolerant to dexamethasone than 3132.

The tolerance for doxorubicin and vincristine were similar between the human and canine cell lines. The Doxorubicin IC50 for 3132 was 0.04 μM and 0.05 μM for Jurkat. The Vincristine IC50 was 2 nM for 3132 and 1.5 nM for Jurkat.

3.5.2. IC50 for drug cocktail

The effect of these drugs, in combination, on the cell viability of 3132 and Jurkat cells was determined after the treatment using 11 different and increasing cocktails of drugs (Figure 5) at the indicated doses (Table 1).

Figure 5. Chemosensitivity Assay to determine the IC50 for drug cocktail.



Source: Garnica, T. K. (2020).

Table 1. Cocktail concentration for Jurkat and 3132 cell line.

	Dexamethasone	Doxorubicin	Vincristine
1	0	0	0
2	0.01 nM	0.5 nM	0.01 nM
3	0.05 nM	1 nM	0.05 nM
4	0.1 nM	5 nM	0.1 nM
5	0.5 nM	0.01 μM	0.5 nM
6	1 nM	0.05 μM	1 nM
7	5 nM	0.1 μM	2.5 nM
8	0.01 μM	0.5 μM	5 nM
9	0.05 μM	1 μM	10 nM
10	0.1 μM	5 μM	15 nM
11	0.5 μM	10 μM	30 nM

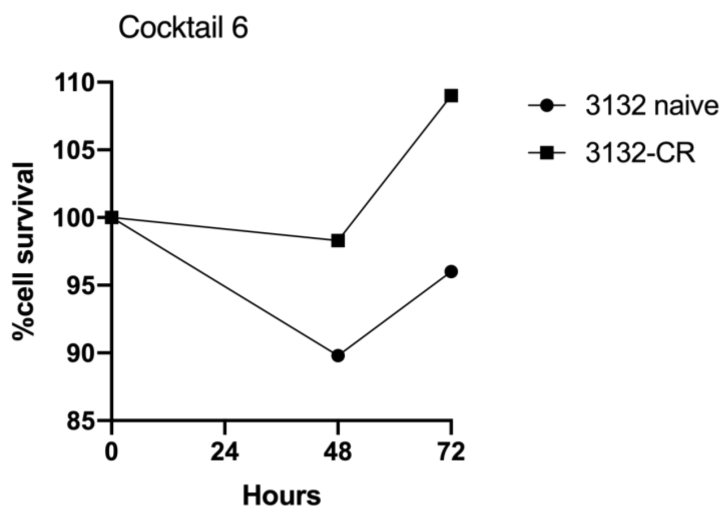
Source: Garnica, T. K. (2020).

This result demonstrated that combination drugs of cocktail 6 can be selected as the IC50 cocktail dose for both cell lines.

3.5.3. The assessment of chemo-resistant phenotype

Cells were incubated with increasing doses of the cocktail for 10 passages. To determine if these cells were chemoresistant, 3132-CR and Jurkat-CR were treated with cocktail 6 and cell viability was determined. We chose the cocktail 6 as potential IC50 cocktail for both cell lines. The naïve cell lines were used as negative controls.

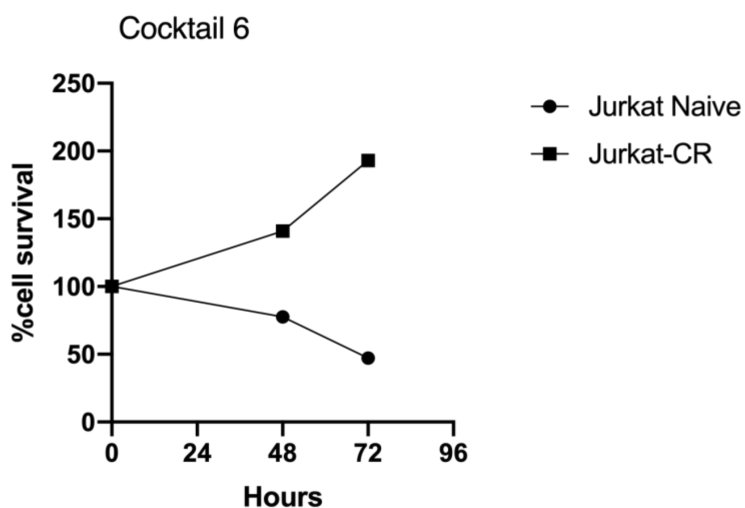
Figure 6. 3132 naïve and CR after cocktail 6



Source: Garnica, T. K. (2020).

3132 had similar results for naïve and chemoresistant. The cocktail 6 killed less than 10% of naïve cells and didn't working as IC50.

Figure 7. Jurkat naïve and CR after cocktail 6.



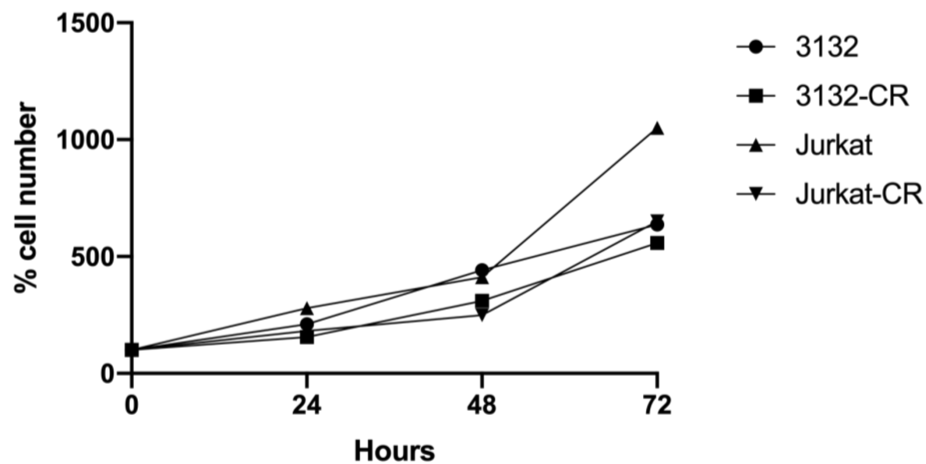
Source: Garnica, T. K. (2020).

For Jurkat cell the cocktail 6 as IC50 worked and the chemo-resistant cells didn't die after treatment while the naïve cells started to die as expected indicating that Jurkat cells were chemo-resistant.

3.5.4. Doubling-time

The doubling-time was assessed after obtained the chemo-resistant cell lines.

Figure 8. Doubling-time for 3132 and Jurkat.



Source: Garnica, T. K. (2020).

The results showed that chemo-resistant cell lines proliferate slower than naïve cells. The doubling-time was: 26.96 hours for 3132 naïve, 29.04 hours for 3132-CR, 21.2 hours for Jurkat naïve and 26.78 hours for Jurkat-CR.

Table 2. Doubling-time for 3132 and Jurkat

Cell line	Time (h)
3132 naïve	26.96
3132-CR	29.04
Jurkat naïve	21.20
Jurkat-CR	26.78

Source: Garnica, T. K. (2020).

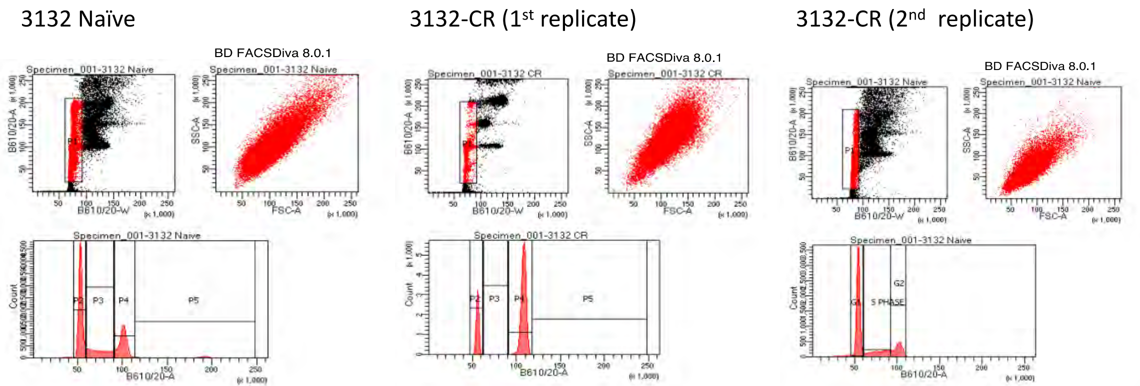
This result is in accordance to the literature and some studies hypotheses that slow-cycling cells are better able to survive treatment, and thus more capable of leading to tumor recurrence (MOORE; HOUGHTON; LYLE, 2012). The slow-cycling cancer cells are also associated to the cancer stem cells (CSC) described in some tumors and responsible for the tumor burden after therapy and tumor dormancy (DE ANGELIS et al., 2019).

3.5.5. Cell Cycle Assay

The results about doubling-time was performed after obtained the chemo-resistant cell lines and were repeated for 2 times. The cells were not synchronized.

The results obtained with 3132 cell lines showed differences between naïve and chemo-resistant cells. The normal cell cycle has the majority of cells at G1 and G2 phase and an intermediary number at S-phase. This first replicate didn't show any cells at S-phase for 3132-CR. Despite the difference this assay was repeated for 3132 cell lines to confirm the difference. The results about second replicate confirmed that 3132 naïve and chemo-resistant didn't have any differences in cell cycle (Figure 9).

Figure 9. 3132 naïve and chemo-resistant cell cycle assays.

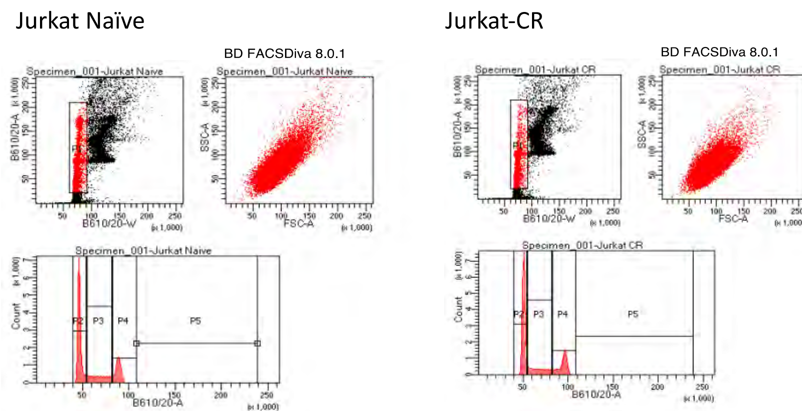


Cell line	G1	S-phase	G2
3132 naïve	33.4%	14.7%	19.6%
3132-CR (1st replicate)	19.5%	0.5%	59.2%
3132-CR (2nd replicate)	35.9%	12.9%	11.9%

Source: Garnica, T. K. (2020).

The Jurkat cell line didn't show any difference between naïve and chemo-resistant cells. The assay was not repeat for Jurkat. The results are attached below (Figure 10).

Figure 10. Jurkat naïve and chemo-resistant cell cycle assay.



Cell line	G1	S-phase	G2
Jurkat naïve	43.7%	18.2%	17.9%
Jurkat-CR	42.7%	17.5%	21.4%

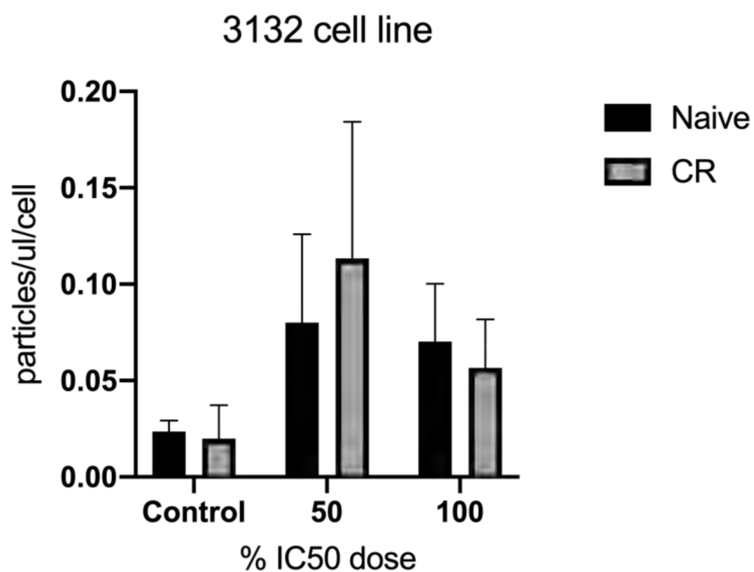
Source: Garnica, T. K. (2020).

Some studies in the literature showed differences in cell cycle between resistant and non-resistant cancer cells. Our results were obtained from just one replicate, so we cannot certainly consider that there is no difference between CR and naïve cells. In addiction we need to test if the chemo-resistant phenotype is a temporary feature, or if heritable changes occur that render all surviving cells permanently resistant to the drugs (ARORA; SPENCER, 2017).

3.5.6. Exosome releases after chemotherapy

This experiment was proposed to evaluate differences in exosomes concentration between chemo-resistant and naïve cells 24 hours after chemotherapy. The graphs below show the number of particles produced.

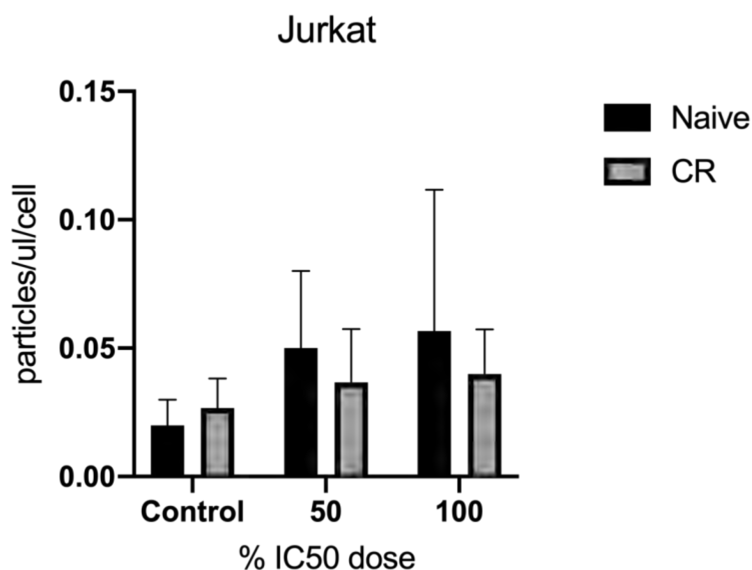
Figure 15. 3132 exosomes released 24 hours after chemotherapy.



Source: Garnica, T. K. (2020).

There were no differences in exosomes concentration between 3132 naïve and 3132-CR (P=0.7661) Two-way ANOVA. The exosomes concentration was different according to control and IC50 doses (P=0.0187) Two-way ANOVA. There was no difference between 50% of IC50 and 100% of IC50 treatment (P=0.7426) T-test. The 50% of IC50 treatment released higher exosome concentration for both cells.

Figure 16. Jurkat exosomes release after chemotherapy.



Source: Garnica, T. K. (2020).

There were no differences in exosomes concentration between Jurkat naïve x Jurkat-CR (P=0.5744) Two-way ANOVA. No differences in exosomes concentration according to control and IC50 doses (P=0.3119) Two-way ANOVA. There was no difference between 50% of IC50 and 100% of IC50 treatment (P=0.0704) T-test.

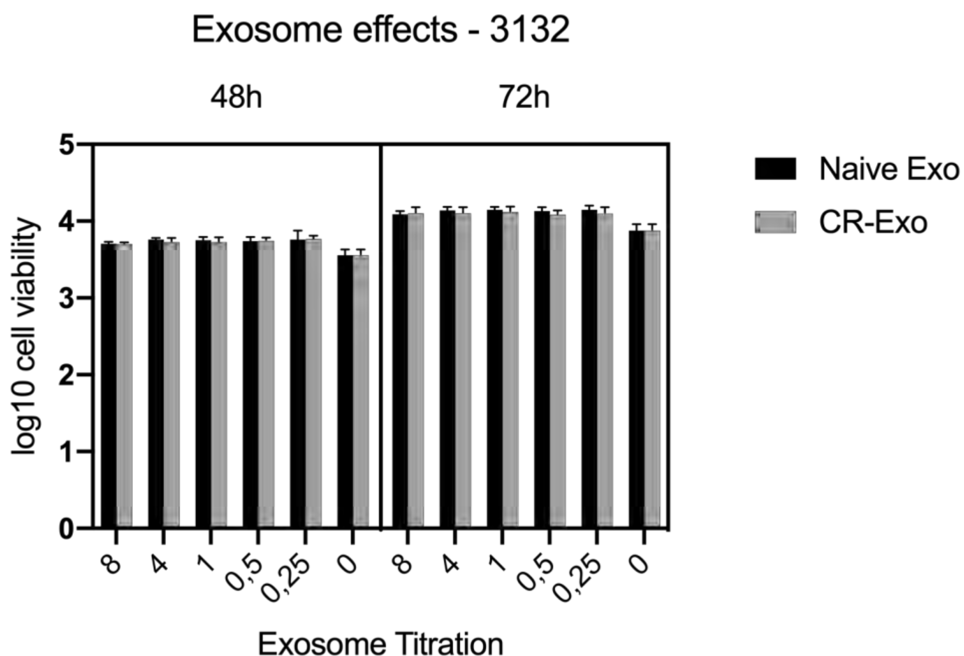
According to literature the chemotherapy can enhance the amount of exosomes release by cancer cells (HONG et al., 2019) (EMAM et al., 2018). This increase in exosome secretion can be correlate with the cellular death caused by chemotherapy and also with the exosome-mediated shuttling of anti-cancer drugs out of the tumor cell (YOUSAFZAI et al., 2018). The 3132 cells secreted more exosomes after the chemotherapy comparing to the control (media) as expected. The Jurkat cells didn’t exhibit differences in exosomes secretion between treated and control but we can observe a tendency of Jurkat-CR in produce less exosomes than Jurkat naïve after chemotherapy. Further assays to show the exosomes

uptake by cells can also bring information about differences in chemo-resistant and naïve cells.

3.5.7. Treatment of Naïve cells using exosomes derived from chemo-resistant cell lines

To test exosome's potential to increase cell proliferation, 3132 and Jurkat naïve cell lines were treated with different exosomes titrations derived from naïve and chemo-resistant cells and analyzed after 48 and 72 hours of treatment.

Figure 17. Exosomes treatment effect on 3132 cells.

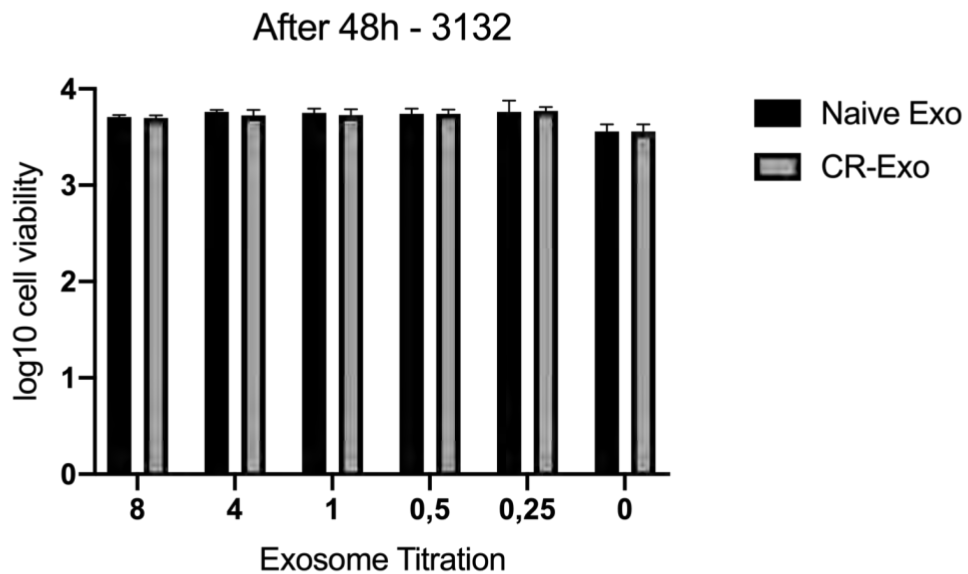


Source: Garnica, T. K. (2020).

The exosomes origin (Naïve and CR) didn't show difference in cellular proliferation for 3132 cells ($P=0.11$) Three-Way ANOVA. The exosomes titration showed difference in 3132 cells proliferation ($P<0.001$) Three-Way ANOVA. The

increase in proliferation was higher after 72 hours of treatment (P<0.001) Three-Way ANOVA.

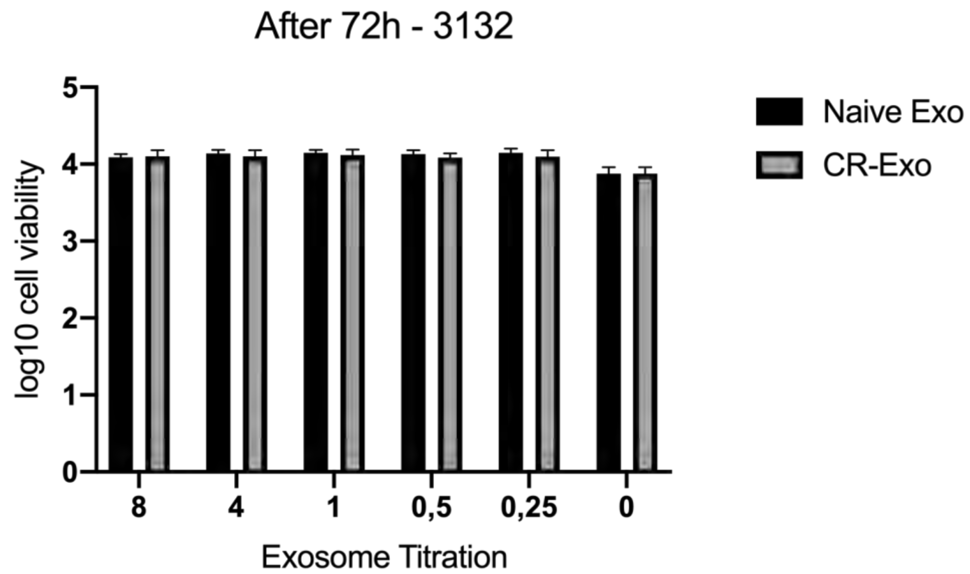
Figure 18. 3132 cell viability after 48 hours of exosome treatment.



Source: Garnica, T. K. (2020).

After 48 hours the origin of exosomes didn't show difference in 3132 cell proliferation (P=0.51) Two-way ANOVA. However, the exosome titration showed differences (P<0.001) Two-way ANOVA.

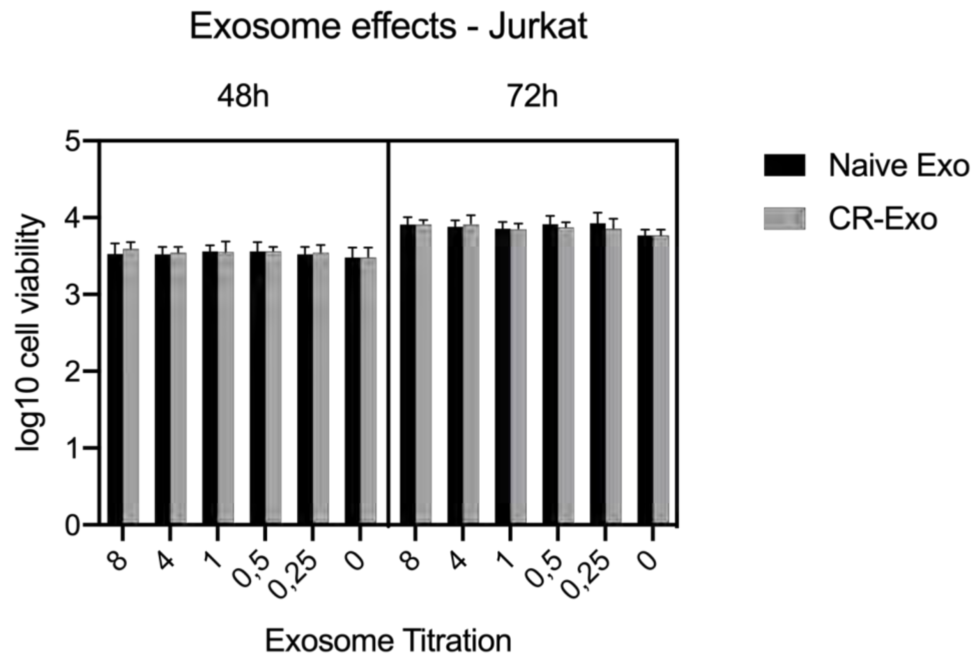
Figure 19. 3132 cell viability after 48 hours of exosome treatment.



Source: Garnica, T. K. (2020).

After 72 hours the exosome origin didn't showed difference in 3132 cells proliferation (P=0.12) but the exosome titration showed (P<0.001).

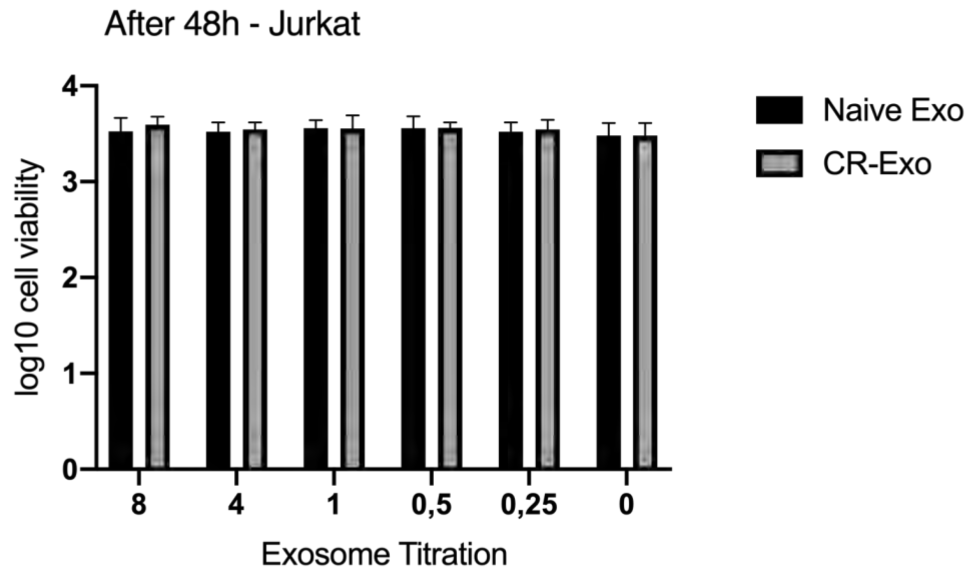
Figure 20. Exosomes treatment effect on Jurkat cell line.



Source: Garnica, T. K. (2020).

The origin of exosomes didn't have difference on Jurkat cells proliferation (P= 0.91) Three-Way ANOVA. The exosomes titration had difference on cell proliferation (P<0.0035). The proliferation was higher after 72 hours (P<0.001).

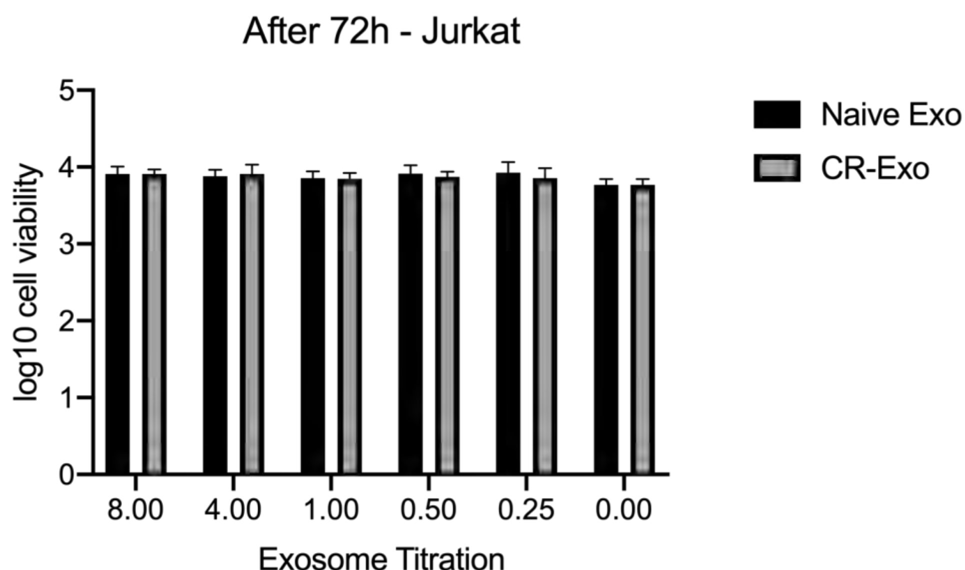
Figure 21. Jurkat cell viability after 48 hours of exosome treatment.



Source: Garnica, T. K. (2020).

After 48 hours of treatment the exosome origin didn't show difference on Jurkat cell proliferation (P=0.46) Two-way ANOVA. The exosome titrations also didn't show difference on Jurkat cell proliferation (P=0.45) Two-way ANOVA.

Figure 22. Jurkat cell viability after 72 hours of exosome treatment.



Source: Garnica, T. K. (2020).

After 72 hours of treatment the exosome origin didn't show difference on Jurkat cell proliferation (P=0.51) Two-way ANOVA. The exosome titrations showed difference on Jurkat cell proliferation (P=0.005) Two-way ANOVA.

3.6. DISCUSSION

According to the literature exosomes derived from chemo-resistant cells can transfer molecules and induce chemoresistance in natural cells (non-resistant). This effect was showed in different cancer types such as breast cancer, prostate and others (SANTOS et al., 2018; HUANG et al., 2019). Our results showed that exosomes from chemo-resistant and from naïve cells have the same effect in cellular proliferation. The exosomes titration has no significative difference on increasing proliferation. Further assays will be needed to assess the effect of exosomes origin on invasion/migration capability.

The main challenge for project execution was the generation of chemo-resistance cells that took 2 months. We also had some problems involving 3132 cell line. The chemosensitivity assay to determine the drugs cocktail IC50 didn't work as expected for 3132 cell line and we could not evaluate the chemo-resistant potential. In addiction a recently study published that 3132 cell line, originally classified as a B-cell lymphoma, was reclassified as a histiocytic sarcoma based on characteristic cytogenomic properties (ROODE et al., 2016). This change on 3132 cells phenotype has direct implication on our results showing that a new canine lymphoma cell line should be chosen for the future experiments. The last experiment purposed was not completed and the evaluation of increase on invasion and migration of naïve cells after treatment with exosomes from chemo-resistant was not perform.

The main objective was to investigate the exosomes importance on transfer chemo-resistant phenotype in canine and human hematological cancers. Our results showed so far that exosomes from chemo-resistant cells and exosomes from naïve cells have the same effect on cells proliferation (at least in Jurkat cells). Further experiments will be needed to elucidate and test the hypothesis.

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4. CONCLUSÃO GERAL

Diante dos resultados obtidos no primeiro estudo podemos concluir que a concentração sérica de VEP no momento do diagnóstico de linfoma multicêntrico em cães pode prever a resposta terapêutica ao CHOP. O subestadiamento e a idade dos cães está relacionada a resposta ao tratamento. A expressão de miR-20a, miR-205 e miR-222 nas VEP pode prever pacientes que terão remissão completa, enquanto a expressão de miR-93 pode prever quais cães não responderam ao CHOP. Com os resultados do segundo estudo concluímos que o tratamento com exossomos provenientes de células quimioresistentes e células nativas possuem o mesmo efeito na proliferação celular.