Universidad de Caldas

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TESIS QUE PRESENTA **ALEJANDRO CLAVIJO MALDONADO**
PARA OBTENER EL GRADO DE MAGISTER EN CIENCIAS BIOLÓGICAS

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DIRIGIDA POR **FREDY ARVEY RIVERA PÁEZ,** *Ph.D*
CODIRIGIDA POR **CARLOS VARGAS HERNÁNDEZ**, *Ph.D*

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	Nombre	Firma
Director	Fredy Arvey Rivera Páez, *Ph.D*	
Co-director	Carlos Vargas Hernández, *Ph.D*	
Jurado		

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A mi esposa e hijo quienes por su apoyo y presencia representan el aliciente de Dios.

DECLARACIÓN DE ORIGINALIDAD

Excepto cuando es explícitamente indicado en el texto, el trabajo de investigación contenido en esta tesis fue efectuado por Alejandro Clavijo Maldonado como estudiante de la Maestría en Ciencias Biológicas entre (agosto) de (2015) y (junio) de (2020), bajo la supervisión y orientación del Dr. Fredy Arvey Rivera Páez y el Dr. Carlos Vargas Hernández.

Las investigaciones reportadas en esta tesis no han sido utilizadas anteriormente para obtener otros grados académicos, ni serán utilizadas para tales fines en el futuro.

Candidato:	Nombre	Alejandro Clavijo Maldonado
Director de tesis:	Nombre	Fredy Arvey Rivera Páez, *Ph.D*
Co-director de tesis:	Nombre	Carlos Vargas Hernández, *Ph.D*

Resumen

Se describieron características epidemiológicas de tumores mamarios caninos (TMC) con énfasis en los factores de riesgo que propenden a la presentación de TMC; raza, predisposición genética, edad, historia reproductiva, influencia hormonal, dieta, masa corporal y características clínicas. Se desarrolló una descripción de la prevalencia del TMC en el Municipio de Manizales, Colombia, tomando como base los pacientes registrados en centros de atención veterinaria. Se realizó la identificación de los patrones de la expresión del gen HER-2 en hembras caninas con TMC, empleando suero sanguíneo mediante las técnicas de ensayo por inmunoabsorción ligado a enzima (ELISA) y espectroscopia infrarroja con Transformada de Fourier (FTIR). Para la detección de concentraciones de proteína HER-2/*neu* se emplearon los kit ELISA (canino y humano). La toma de espectros IR se realizó en modo absorbancia con rango de frecuencia de 400-4000 cm^{-1} y resolución de 4 cm^{-1}. El análisis estadístico para la descripción de la prevalencia incluyó prueba de Pearson chi-cuadrado con corrección de Yates y prueba exacta de Fisher (*p-value<0.05*), riesgo relativo, razón de momios (IC95%) y análisis de supervivencia de Kaplan-Meier. Se elaboraron curvas ROC y la estimación de ABC (IC 95%) para la identificación del punto de corte de las concentraciones en suero sanguíneo de HER-2 usando la prueba de ELISA. El análisis de los picos de expresión IR se realizó mediante la estimación de ABC y ARBC. Las razas puras presentaron una prevalencia del 79,14% y en general, la edad de 9,3 años fue el pico de presentación de neoplasia mamaria. Adicionalmente, la dieta casera posee alta relación con la prevalencia de TMC. El patrón clínico de presentación de TMC afectó principalmente el par mamario inguinal y el índice Kaplan-Meier fue mayor para hembras intervenidas quirúrgicamente desde el diagnóstico que las que no fueron intervenidas. El punto de corte óptimo en la prueba ELISA canina fue ≥0.31 ng/ml, Sn de 71.4% (CI 95%: 41,9%-91,6%) y Sp 67% (CI 95%: 22,3%-95,7%). Las AUC para ELISA canina y humana fueron 0,75 y 0,45 respectivamente. Los espectros IR correspondientes a lípidos (1161 cm^{-1}, 1452 cm^{-1}, 2851 cm^{-1}) son de relevancia para la identificación de expresión del gen HER-2. Los hallazgos del presente estudio permitieron discriminar los estados HER/*neu*+ y HER-2/*neu*-, así como las características bioquímicas ligadas a ambos estados.

Palabras clave

ErbB2, Espectro infrarrojo, inmunoabsorción, oncoproteína, neoplasia mamaria.

Abstract

We described the epidemiological characteristics of canine mammary tumors with a focus on the risk factors that promote the presentation of CMT, such as breed, genetic predisposition, age, reproductive history, hormonal influence, diet, body mass, and clinical characteristics. Based on this, we conducted a prevalence description of CMT in the municipality of Manizales, Colombia. The gene expression patterns of HER2 in blood serum collected from female dogs with CMT were determined using enzyme-linked inmunosorbent assay (ELISA) and Fourier-transform infrared spectroscopy (FTIR). The detection of HER2/*neu* protein levels was done using canine and human ELISA kits. Infrared (IR) spectroscopy was done in absorbance mode at a frequency range of 400-4000 cm^{-1} and resolution of 4 cm^{-1}. The statistical analyses performed included Pearson's chi-squared test with Yates' correction and Fisher's exact test (*p-value<0.05*), relative risk, odds ratio (IC95%), and Kaplan-Meier survival analysis. We created ROC curves and estimated the AUC (IC 95%) to identify the ELISA cut-off of HER2 serum levels. The IR analysis of expression peaks was based on the estimation of AUC and RAUC. Purebreds showed a disease prevalence of 79.14% and, in general, the peak of presentation of mammary neoplasia was found at 9.3 years old. Furthermore, a homemade diet was found highly associated with the prevalence of CMT. The clinical presentation of CMT mainly affected the inguinal mammary glands; furthermore, the Kaplan-Meier estimator was greater for females with surgical intervention since diagnosis than for non-intervened females. The optimal cut-off for the canine ELISA tests was ≥0.31 ng/mL with 71.4% sensitivity (CI 95%: 41.9%-91.6%) and 67% specificity (CI 95%: 22.3%-95.7%). AUC for the canine and human ELISA tests were 0.75 and 0.45, respectively. The IR spectra of lipids (1161 cm^{-1}, 1452 cm^{-1}, 2851 cm^{-1}) are relevant to determine HER2 gene expression. The findings of this study allowed discriminating HER/*neu*+ and HER-2/*neu*- states, as well as the biochemical characteristics linked to both states.

Keywords

ErbB2, infrared spectrum, immunoabsorption, oncoprotein, mammary neoplasia.

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1. CAPÍTULO INTRODUCTORIO

La progresión tumoral se caracteriza por el aumento de poblaciones celulares con mecanismos de control y de apoptosis autónomos lo cual implica la acumulación gradual de alteraciones fisiológicas y fenotípicas (Gurova et al. 2002). Según autores como Clamp et al. (2003), Murray (2005), el origen tumoral puede responder a factores hereditarios, ambientales y mecanismos no celulares (p.ej. sistema inmunitario, endocrino, estroma, vascularización). El cáncer mamario (CM) se caracteriza por un alto polimorfismo genético caracterizado por la pérdida o ganancia de información (Hsu et al. 2009).

Cáncer de Seno: Contexto mundial

En el año 2004, *The World Health Organization* (WHO) reportó 7,4 millones de muertes por cáncer, cifra que para el 2005 ascendió a 7,6 millones (WHO, 2006, 2008). Para el año 2012, la mortalidad asociada a cáncer creció a 8,2 millones de muertes (WHO, 2012, 2016). Hacia el año 2015 el incremento de casos fue aproximadamente de 8,8 millones de pacientes (WHO, 2017), elevándose a 9 millones en el año 2016 (WHO, 2018) y posteriormente a 9,6 millones en el año 2018 (WHO-GLOBOCAN, 2018). La WHO, proyecta que en el 2020 el número de muertes causadas por cáncer ascenderá a cerca de 10 millones equivalente una tasa de mortalidad del 529 por cada 1000 pacientes. (Cancer Toorrow, 2018).

El cáncer de seno humano (CSH) es una enfermedad de carácter público (Osma & Uribe, 2013; WHO, 2015). Del global de casos reportados de cáncer en 2008, el CSH fue la causa más común de muerte en mujeres adultas, con un 16% de casos con desenlace fatal (WHO, 2008). En 2012, CSH ocupaba el segundo lugar con 1,7 millones de casos diagnósticados (11,9%), perteneciendo dos tercios de estos a países en vía de desarrollo, y por ende uno de los principales factores de muerte antes de los 70 años de edad (WHO, 2012; Steward & Wild, 2014; WHO, 2016).La IARC (*International Agency for Research on Cancer*) posicionó a CSH en el primer lugar con un 25,2% (WHO-IARC, 2013). En el año 2018, de los 18 millones de casos de cáncer reportados, el CSH representaba una incidencia del 11,6% (WHO-The Global Cancer Observatory, 2019). Se estima que el CSH seguirá ocupando el primer lugar a nivel mundial en mujeres, con una proyección a 3,05 millones de casos en 2040 y una mortalidad de 991 mil pacientes en el mismo (WHO-GLOBOCAN, 2018).

Cáncer de Seno: Contexto nacional

En Colombia, después del cáncer de cuello uterino, el CSH es el principal cáncer que padecen las mujeres (CONPES SOCIAL 161, 2013). Entre los años 2000 a 2006, se reportaron aproximadamente 70887 casos nuevos de cáncer, de los cuales, 38571 casos correspondían a mujeres y la principal ubicación era la mamaria (MINSALUD, 2012). Para el año 2014, el CSH estuvo asociado a 2649 muertes, aparte de 8.686 nuevos casos en las principales ciudades del país (MINSALUD, 2014). En 2018, el incremento de casos reportados fue de 13380, se proyecta un incremento a 14124 casos en 2020 y una estimación de 20957 pacientes con CSH en 2040 (WHO-GLOBOCAN, 2018). El número de muertes estimadas en el año 2018 por CSH fue de 3702 pacientes, para el 2020 se proyectan 3942 y para el 2040, 6600 obedeciendo al crecimiento demográfico (WHO-GLOBOCAN, 2018). Los costos estimados desde el diagnóstico, tratamiento y seguimiento hasta cinco años dependen del tipo de CSH, así los costos de tratamiento más representativos en Colombia están representados en CSH regional con un costo de $65.603.537 y el metastásico con un valor de $144.400.865 y el de menor cuantía es el *in situ* con un costo de $8.996.987 (referencias). Si el paciente tiene una recaída el valor desde el diagnóstico de extensión hasta el seguimiento de cinco años puede ascender hasta a $70.221.062 (Gamboa et al. 2016).

Tumor mamario canino (TMC)

Entre los años 2002-2003, la población total de caninos ascendía a 500 millones aproximadamente (Hsu et al. 2003). En el 2013 alcanzó los 700 millones (Massei & Miller, 2013; Hughes & Macdonald, 2013) y para finales de 2017 llegó a los 900 millones (Atitwa, 2018). La incidencia del cáncer humano y canino ha aumentado en los últimos diez años (Cervone et al. 2019). En este mismo periodo, la lista de tumores caninos tanto malignos como benignos con mayor frecuencia es encabezada por piel - anexos y glándula mamaria, luego, tracto genital, cavidad oral, hígado, sistema hemolinfático y sistema osteomuscular (Šoštarić-Zuckermann et al. 2013; Boerkamp et al. 2014; Baioni et al. 2017; de la Cruz et al. 2017; Pastor et al. 2018).

La glándula mamaria está expuesta a sufrir varios tipos de lesiones neoplásicas y no neoplásicas tales como mastitis, esteatitis, galactostasis, galactorrea, y agalactia (Sangha et al. 2012; de la Cruz et al. 2017). Dentro de las lesiones neoplásicas se encuentran las benignas, malignas y las

no clasificadas (Vidales & Eslava, 2007; Cassali et al. 2011; Goldschmidt et al. 2011). De estas, el TMC) es una de las patologías de mayor importancia en Medicina Veterinaria debido a su frecuencia y complejidad clínica (Zatloukal et al. 2005; Vascellari et al. 2009; Cassali et al. 2011; Sangha et al. 2012; Salas et al. 2015).

TMC – generalidades clínicas

El TMC se puede presentar en hembras enteras e incluso en menor frecuencia en esterilizadas (Sorenmo et al. 2011). Durante la exploración clínica, se debe considerar los linfonodos al igual que ambas líneas de glándulas mamarias, en las cuales a la palpación habitualmente se puede notar masas nodulares de diversos tamaños y movibles asociadas a reacciones inflamatorias localizadas, laceraciones y ulceraciones cutáneas (Cassali et al. 2011). El abordaje clínico, debe ser complementado con placas radiográficas ventro-dorsales, laterolateral izquierda y derecha a fin de determinar la presencia o no de metástasis a nivel pulmonar sin descartar el hígado el cual es el sitio más común de metástasis distante en pacientes con TMC maligno implicando un mal pronóstico (Cassali et al. 2011; Sorenmo et al. 2011).

Como consecuencia se puede definir el estado del tumor que implica el grado de compromiso y la extensión de la afectación y por lo tanto el pronóstico y la conducta terapéutica a seguir (Cruz, 1999). Una herramienta útil en el ejercicio médico es la estadificación clínica la cual consiste en determinar la etapa de desarrollo de cáncer mamario (Edge et al. 2010). El sistema TNM (T=Tumor; N=Nódulo; M=Metástasis) fue desarrollado por la OMS (Organización Mundial de la Salud) el cual permite clasificar los diferentes casos de cáncer en etapas (Owen, 1980) de acuerdo al tamaño tumoral, estado de linfonodo y la presencia de metástasis (Sorenmo et al. 2011) (Tabla 1, Anexo 1). El tamaño tumoral es considerado como un factor pronóstico independiente para CMC donde tamaños iguales o inferiores a 3cm presentan un pronóstico más favorable que los de tamaño 5cm o superior (Ferreira et al. 2009; Cassali et al. 2011; Sorenmo et al. 2011). La evaluación de linfonodos regionales es de gran importancia en la tasa de sobrevida de las pacientes que padecen TMC, donde su relación con patrones de metástasis reducen drásticamente los tiempos de sobrevivencia (Cassali et al. 2011). El drenaje linfático también presenta cambios en presencia de TMC (Tabla 2), lo cual agranda los linfonodos haciéndolos palpables y deben ser evaluados por citología a través de biopsia por aspiración con aguja fina (BAAF) (Sorenmo et al. 2011).

Tabla 1. Sistemas de estadistificación TNM para Tumor Mamario Canino (TMC)

Sistema de estadificación OMS - 1980				Sistema de estadificación OMS - 2007				Sistema de estadificación AJCC - 2015			
	T	**N**	**M**		**T**	**N**	**M**		**T**	**N**	**M**
Estado I	$T_{1a,b,c}$	N_0	M_0	Estado I	T_1	N_0	M_0	Estado I	Tis	N_0	M_0
Estado II	T_0	N_1	M_0	Estado II	T_2	N_0	M_0	Estado IA	T_1	N_0	M_0
	$T_{1a,b,c}$	N_1	M_0	Estado III	T_3	N_0	M_0	Estado IB	T_0	N_{1mi}	M_0
	$T_{2a,b,c}$	N_0 ó N_{1a}	M_0	Estado IV	Cualquier T	N_1*	M_0		T_1*	N_{1mi}	M_0
Estado III	$T_{3a,b,c}$	Cualquier N	M_0	Estado V	Cualquier T	Cualquier N	M_1	Estado IIA	T_0	N_1**	M_0
	Cualquier T	Cualquier N_b*	M_0						T_1	N_1	M_0
Estado IV	Cualquier T	Cualquier N	M_1						T_2*	N_0	M_0
								Estado IIB	T_2	N_1	M_0
									T_3*	N_0	M_0
								Estado IIIA	T_0	N_2*	M_0
									T_1	N_2	M_0
									T_2	N_2	M_0
									T_3	N_1	M_0
									T_3	N_2	M_0
								Estado IIIB	T4*	N_0	M_0
									T_4	N_1	M_0
									T_4	N_2	M_0
								Estado IIIC	Cualquier T	N_3	M_0
								Estado IV	Cualquier T	Cualquier N	M_1

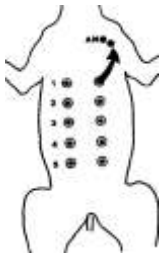
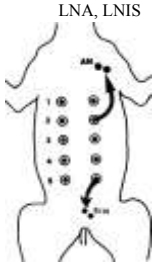
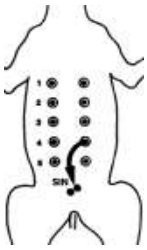
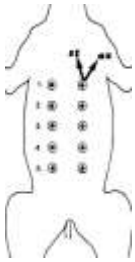
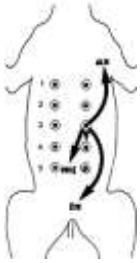
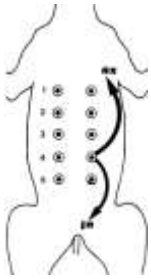
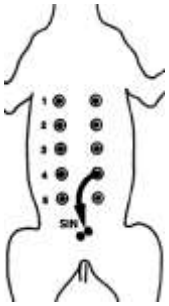
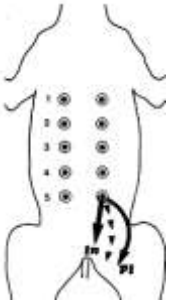
Abreviaturas

T: Tumor primario	a: no fijo	Tis: Carcinoma in situ
T0: Tumor no evidente	b: fijo en piel	T1*: < 2cm de diámetro
T1: < 3cm de diámetro	c: fijo en músculo	T2*: 2-5cm de diámetro
T2: 3-5cm de diámetro	b*: fijo	T3*: > 5cm de diámetro
T3: > 5cm de diámetro		T4*: Cualquier dimensión con adherencia a pared del tórax y/o piel (ulceración o nódulos cutáneos)
T4: cualquier T, carcinoma inflamatorio		
N: Estado de linfonodo regional		N1**: metástasis ipsilateral, linfonodo axilar
N0: sin Metástasis		N2*: metástasis ipsilateral, linfonodo axilar fijo o enmarañado
N1: Linfonodo - metástasis ipsilateral		N3: metástasis ipsilateral infraclavicular
N1*: metástasis presente		
N2: Linfonodos - metástasis bilateral		
M0: sin metástasis distante		
M1: metástasis distante		

Owen, 1980; Rutteman & Withrow, 2007; Edge et al. 2015

Como apoyo al proceso diagnóstico se han sugerido sistemas de clasificación de tumores mamarios en caninos, Hampe & Misdorp (1974) realizaron un proceso de clasificación basado en aspectos morfológicos basados en la clasificación de años anteriores de la OMS (Scarff & Torloni, 1968). Varios sistemas de clasificación han sido sugeridos desde entonces, pero la más ampliamente adoptada es la establecida por Misdorp et al. (1999) con un grado de detalle aun mayor al apoyarse de características nucleares de las células y detalles histológicos. A partir de este sistema, se han propuesto otras dos clasificaciones en trabajos paralelos propuestos por Goldschmidt et al. (2011) más amplio al crear nuevas categorías por apoyo inmunohistoquímico y Cassali et al. (2011) que propone una recategorización al sistema de 1999 (Anexo 2).

Tabla 2. Drenaje linfático normal y neoplásico en hembras caninas

Glándula mamaria	PM1 - Craneal Torácico	PM2 - Caudal Torácico	PM3 - Craneal Abdominal	PM4 - Caudal Abdominal
Drenaje linfático normal[1, 2]	LNA 	LNA, LNIS 	LNA, LNIS 	LNIS 
Drenaje linfático neoplasico[3]	LNA, LNE 	LNA, LNE 	LNA, LNIM, LNIS 	LNA, LNIS 
Glándula mamaria	PM5 - Inguinal			
Drenaje linfático normal[1]	LNIS 			
Drenaje linfático neoplasico[3]	LNIS, LNP 			

LNA: Linfonodo axilar

LNE: Linfonodo esternal

LNIM: Linfonodo iliaco medial

LNIS: Linfonodo inguinal superficial

LNP: Linfonodo popliteo

Normalmente, el primer par de glándulas mamarias poseen drenaje linfático hacia los nódulos axilares y en algunos casos puede drenar hacia los cervicales, entre tanto que el segundo hacia los nódulos axilares. El tercer par, simultáneamente, drena hacia el nodo axilar superficial y el inguinal. El cuarto par drena hacia los nódulos inguinales superficiales, aunque también lo puede hacer hacia los nodos iliacos mediales al mismo tiempo, entre tanto que el quinto par drena hacia los nodos inguinales superficiales. Sin embargo, bajo la presencia natural de CMC, se ha demostrado cambios en el drenaje linfático, de tal modo que el primer y segundo par drenan simultáneamente hacia el LN (linfonodo) axilar y esternal simultáneamente, tercer par hacia LN axilar superficial ipsilateral y LN iliaco medial, diferente al cuarto par que es solo causal, LN superficial inguinal y por ultimo el quinto par drena hacia nódulo superficial inguinal aunque también lo puede hacer hacia el LN popliteo ipsilateral.

[1]Patsikas et al. 1996a; [2]Patsikas et al. 1996a; [3]Patsikas et al. 2006

Biomarcadores en el estudio de alteraciones neoplásicas mamarias

Se han desarrollado diversas técnicas para el estudio de neoplasias mamarias, entre ellas la detección de biomarcadores como las proteínas las cuales, permiten obtener información sobre la salud del tejido, grado de riesgo, ofrece directrices precisas para el desarrollo de tratamientos y el establecimiento pronóstico (Kreunin et al. 2007; Jesneck et al. 2009). La investigación de biomarcadores con valor pronóstico de cáncer implica empleo de biopsias lo cual no siempre es posible (Jesneck et al. 2009). Por lo tanto, se han desarrollado métodos con suero sanguíneo y plasma los cuales han sido aplicados en el estudio de CSH (Jesneck et al. 2009; Savino et al. 2009) como también en TMC (Campos et al. 2015).

Uno de los biomarcadores de mayor importancia en CSH, es el gen del crecimiento epidérmico humano tipo II (HER-2), el cual consiste de una proteína de 185-kilodaltons (kDa) (Akiyama et al. 1986). En mujeres se ha encontrado que la amplificación de la proteína HER-2/ *neu* (c-erbB-2/*neu*) oscila entre el 20 – 30% e implica un mal pronóstico con un alto impacto en la sobrevivencia de las pacientes (Andrulis et al. 1998; Andrade & Harris 2002; Di Gioia et al. 2015). En caninos, la expresión de la oncoproteína HER-2/ *neu* en carcinoma mamario, sugiere la participación de la proteína en la carcinogénesis constituyéndose en indicador de utilidad en el pronóstico (Rungsipipat et al. 1999; de las Mulas et al. 2003; Antuofermo et al. 2007). Sin embargo, en estudios más recientes controvierten esta posición ya que la sobreexpresión de HER-2/*neu* en tumores malignos y benignos, no presenta diferencias significativas (Ressel et al. 2013; Burrai et al. 2015) ni tampoco se asocia con patrones de metástasis ni porcentaje de sobrevivencia de hembras con CMC (Campos et al. 2015). Por lo tanto, aun no es clara la dinámica de la expresión de HER-2 en la progresión tumoral ni los patrones del gen en diferentes tipos de CMC (Ressel et al. 2013; Burrai et al. 2015).

La presente investigación describe los factores de riesgo de CMC y la importancia clínica del gen HER-2. Así mismo, se realizó una aproximación al estado actual de la prevalencia de CMC en el municipio de Manizales, y finalmente, se evaluó la factibilidad, sensibilidad y especificidad de las técnicas Ensayo por Inmunoabsorción Ligado a Enzimas (ELISA) y Espectroscopia Infrarroja con Transformada de Fourier (FTIR), como medios de estudio de características de la expresión de HER-2 en hembras caninas a partir de suero sanguíneo.

2. OBJETIVOS

Objetivo general

Evaluar el gen HER-2 como biomarcador en suero sanguíneo en hembras caninas con tumor mamario en Manizales-Colombia.

Objetivos específicos

Describir las características de la prevalencia del cáncer mamario canino en el Municipio de Manizales, Colombia.

Cuantificar niveles de proteína asociada al gen HER-2 a través de la detección en suero sanguíneo comparando dos kit de Ensayo por Inmunoabsorción Ligado a Enzimas - ELISA.

Evaluar espectros de expresión de HER-2 en suero sanguíneo de hembras caninas a través del uso de Espectroscopia Infrarroja Transformada de Fourier - FTIR.

3. MATERIALES Y MÉTODOS

3.1. Área de influencia del estudio

El estudio se realizó en el Municipio de Manizales (Caldas – Colombia), durante los años 2017-2019. En total ocho centros de atención veterinaria estuvieron involucrados en el desarrollo mediante el suministro de información clínica y muestras sanguíneas de hembras caninas. Para la construcción de la base de datos de pacientes realizó la solicitud de autorización ante centros de atención veterinaria y fundaciones para acceso a la casuística de las historias clínicas y la identificación de hembras para su inclusión en el proyecto, por lo cual se expidió el consentimiento informado a los propietarios para la posterior toma de muestras (Anexo 3).

3.2. Población objetivo y análisis epidemiológico

Se construyó una base de datos de 15961 pacientes registrados en centros de atención veterinaria, correspondiente al periodo comprendido entre 2014 y 2017. Las variables analizadas correspondieron a: ubicación geográfica, raza (miniatura, pequeña, mediana, grande, gigante) y tipo de raza (puro o cruce), edad, peso, estado reproductivo, tratamientos hormonales preliminares, caracterización del tumor (tipo, tamaño, total de glándulas mamarias afectadas, ubicación, desarrollo de metástasis), supervivencia para lo cual se realizaron llamadas telefónicas a los propietarios.

3.3. Variables de estudio

El acceso a las pacientes se realizó con previa autorización de los propietarios. Para la recolección y análisis de la información se adaptó el formato *Cancer Staging Form* (*UNIVERSAL*) - propuesto por el Departamento de Medicina y Cirugía de Pequeños Animales, Colegio de Medicina Veterinaria de la Universidad de Missouri (Henry & Higginbotham 2010).

Los factores tenidos en cuenta para análisis y seguimiento de pacientes diagnosticadas con CMC, fueron la edad, raza, estado reproductivo, intervención quirúrgica, medicación relacionada (tratamientos hormonales), número y dimensión de las lesiones, presencia o no de ulceración, adherencias, necrosis y calcificación.

Con fines estadísticos y de análisis se tuvo en cuenta edad al inicio de la patología (en años de vida), peso (kilogramos), raza y tipo de raza, diámetro del tumor (centímetros), ubicación de la

glándula mamaria y ubicación de la afectación (par torácico craneal y causal, abdominal craneal y causal y par inguinal), reporte diagnóstico, ovariohisterectomía (si/no), número de tumores (único, múltiple), tipo de procedimiento quirúrgico empleado (si/no), metástasis en linfonodo (si, no), fallecimiento (incluye animales sanos, muerte por complicación tumoral), tiempo de desarrollo de la patología, desde inicio de patología hasta intervención quirúrgica; ≤ 6 meses, > 6 a 12 meses, > 12 meses (Anexo 4).

3.4. Pacientes y muestras

Las muestras se obtuvieron de hembras diagnósticadas con TMC (n=14) y hembras sanas (n=6). Los procedimientos de toma y manejo de muestras de sangre se realizaron siguiendo el procedimiento operativo estandarizado para la recolección de suero y plasma (Tuck et al. 2009). Por individuo, se colectaron aproximadamente 4 ml de sangre obtenida mediante punción venosa aséptica, la cual se almacenó en tubos vacutainer sin anticoagulante tapa roja (BD Vacutainer, Becton Drive). Los contenedores de colecta se codificaron individualmente. Cada muestra se dejó por un periodo de tiempo entre 30 a 60 minutos a temperatura ambiente en posición vertical a fin de lograr la contracción del coagulo sanguíneo y exudado del suero. El transporte refrigerado no se realizó hasta la formación de los coágulos en los tubos de colecta a causa de los posibles golpes sufridos entre las células del coagulo, provocando así la rotura celular y por ende hemólisis (Lippi et al. 2008).

Posteriormente, cada una de las muestras se sometió a centrifugación de 2500 a 3000 rpm durante 10 minutos. La separación se realizó en las dos horas siguientes de la toma de muestra a fin de evitar el intercambio de compuestos entre las células y demás elementos formes de la sangre y su deterioro. La centrifugación se realizó hasta que el coagulo estuviera completamente formado a fin de permitir una separación adecuada de las fases.

Se tomó el suero con la ayuda de una micropipeta estéril de 200 µl volumen. Normalmente, de 1 ml de sangre se pueden obtener 300 µl de suero. Siguiendo la metodología de varios autores (Chase et al. 2012; Campos et al. 2015; Macotpet et al. 2020), el suero obtenido se separó en alícuotas en tubos eppendorf o crioviales correspondientes a las pruebas de ELISA y FTIR, las cuales se almacenaron a temperatura de -80°C evitando ciclos de descongelación - congelación a fin de evitar la lisis u otro tipo de deterioro. Adicionalmente, se tomaron muestras de tejido

mamario de pacientes que fueron sometidas a mastectomía. Se realizaron placas histológicas, coloreadas con hematoxilina-eosina (H&E).

3.5. Detección y medición de proteína HER-2 en suero sanguíneo

3.5.1. Análisis de ELISA

Para detección y cuantificación de la proteína de HER-2 en suero se empleó el *kit Canine Epidermal Growth* Factor Receptor 2 (Her2Ab) ELISA Kit referencia MBS2606515 (MyBioSource, Southern California, San Diego - USA) y *Human* HER-2 Platinum ELISA referencia BMS207-2 (eBioscience, Vienna, Austria). La medición se realizó por espectrofotometría en lector de microplatos Rayto RT-2600c (Guangming, Shenzhen, China) a una longitud de onda de 450 nm. Los patrones con concentraciones conocidas (incluidos en cada kit) y las muestras se prepararon por duplicado siguiendo las indicaciones de los fabricantes.

3.5.2. Análisis FT-IR

Cada criovial debidamente rotulado contenía 40 µl de los cuales fue empleado por cada lectura 5µl de suero con un tiempo de descongelación de 10 minutos antes de la lectura a temperatura ambiente. Para la toma de los espectros IR (infrarrojos) se empleó un espectrómetro Alpha ATR Platinum Bruker. Las lecturas se realizaron bajo condiciones óptimas de 20°C temperatura en sala y 40% humedad relativa. Adaptando la metodología de Elmi et al. (2017), el modo de expresión de la lectura fue en absorbancia bajo un rango de frecuencia de 400 a 4000 cm^{-1} y resolución de 4 cm^{-1}, 50 escaneos por muestra analizada durante aproximadamente 45 segundos.

3.6. Análisis de datos

Se realizó un proceso de clasificación de la base de datos suministrada por los ocho centros de atención veterinaria. Las variables tenidas en cuenta para el estudio y que hacen parte de la estructura de las historias clínicas fueron: raza, edad, tamaño corporal de acuerdo a la raza, estado reproductivo, tipo de dieta y diagnostico (sana – TMC[1]).

La relación entre las variables raza, edad, peso, tipo de alimentación y estado reproductivo fue determinado usando la prueba de Pearson's chi-cuadrado con corrección continua de Yates y

[1] Se define TMC ya que hubo pacientes con hiperplasia mamaria.

prueba exacta de Fisher's para datos continuos con *p-value*<0.05. Para las hembras de seis años en adelante diagnosticadas con TMC, se estimó la tasa de incidencia (Riesgo Relativo - RR) al igual que la Razón de Momios (Odds Ratio-OR) con un intervalo de confianza (IC) del 95%, para las variables anteriormente mencionadas. Los datos fueron analizados en el software R v.3.5.3. Para análisis de sobrevivencia entre hembras con y sin intervención quirúrgica a partir del diagnóstico de TMC, se empleó el estadístico Kaplan-Meier (*p-value*<0.05) con el software GraphPad Prism v.8.2.0, GraphPad Software, Inc., San Diego, CA (Motulsky, 2016).

La curva ROC (*Receiver Operating Characteristic*) ilustra la sensibilidad y especificidad de cada uno de los probables puntos de corte de una prueba diagnóstica en la cual, la escala de medición es continua. El punto de corte óptimo de concentración en suero sanguíneo de HER-2/*neu* mediante el análisis de ELISA, se estimó a través de la mayor exactitud, mayor sensibilidad (Sn), especificidad (Sp) e índice de Youden (J) y menor distancia a la esquina para ambos kit de ELISA. Para cada curva ROC fue estimada las áreas bajo la curva (ABC), a fin de identificar la capacidad discriminativa de cada prueba.

El punto de corte de ambas pruebas fue empleado como criterio pronóstico para distribuir las pacientes fueron clasificadas en TMC con medición sérica de HER-2 igual o mayor al punto de corte, "verdadero positivo" (TP = TMC - HER-2/*neu*+); pacientes control con medición sérica de HER-2 inferior al punto de corte, "verdadero negativo" (TN = sanas - HER-2/*neu*-); pacientes con TMC con medición sérica de HER-2 menor al punto de corte, "falso negativo" (FN = TMC - HER-2/*neu*-) y pacientes control con medición sérica de HER-2 igual o mayor al punto de corte, "falso positivo" (FP = sanas - HER-2/*neu*+). De acuerdo al criterio pronóstico por cada prueba se determinó el valor predictivo positivo (VPP) negativo (VPN).

Los espectros originales se analizaron con el software OriginPro 9.0.0 (OriginLab Corporation, Northampton, MA 01060 USA). Cada uno fue normalizado con corrección de la línea base, a través de un filtro basado en el algoritmo Savitzky–Golay, usando un orden polinomial de segundo grado. Las regiones seleccionadas para su análisis fueron 800 cm^{-1} a 1800 cm^{-1}, constituida por 800 cm^{-1} a 1100 cm^{-1}, 1100 cm^{-1} a 1380 cm^{-1}, 1380 cm^{-1} a 1700cm^{-1}, 1700 cm^{-1} a 1800 cm^{-1}, y la región 2800 cm^{-1} a 3000 cm^{-1}, los cuales se analizaron a través de la segunda derivada y se estimó la AUC para cada espectro (original y segunda derivada). La proporción entre ABC fue descrita mediante áreas relativas bajo la curva (ARBC). Adicionalmente, se identificaron los grupos funcionales y los modos vibracionales de acuerdo a resultados de

investigaciones anteriores (Bi et al. 2014; Gavgiotaki et al. 2016; Elmi et al.2017; Ferreira et al. 2020).

Se realizó prueba de normalidad Shapiro-Wilk y pruebas paramétricas para distribuciones normales y no paramétricas para distribuciones sin normalidad para los valores de las absorbancias en las pruebas ELISA y los espectros IR. Un IC de 0.95 y un p-valor <0.05 fue considerado como estadísticamente significativo. Los análisis estadísticos se realizaron utilizando R v.3.2.2, NCSS 11 (NCSS, Kaysville, Utah, USA) y OriginPro 9.0.0.

Esta investigación adoptó las consideraciones bioéticas en investigación en salud, amparada en el Estatuto nacional de protección de los animales (Ley 84 de 1989), "Por la cual se adopta el Estatuto Nacional de Protección de los Animales y se crean unas contravenciones y se regula lo referente a su procedimiento y competencia", la Resolución No. 008430 de octubre de 1993 de la Republica de Colombia – Ministerio de Salud "Por la cual se establecen las normas científicas, técnicas y administrativas para la investigación en salud", específicamente en Título V "La Investigación Biomédica con Animales" y obedeciendo a la Ley 576 de 2000 "Código de Ética para el ejercicio profesional de la medicina veterinaria, la medicina veterinaria y zootecnia y zootecnia ", por lo cual se pide autorización y posterior aprobación del Comité de Ética para Experimentación con Animales (CEEA) de la Vicerrectoría de Investigaciones y Postgrados de la Universidad de Caldas, Acta 3 de 2016 con código de aprobación número 15061601, con notificación de 01 de junio de 2016 (Anexo 5).

4. RESULTADOS

Los resultados se presentan a manera de capítulos. Cada capítulo consta de un artículo científico, publicado o en preparación en revista internacional especializada en el área estudio.

Capítulo 1.

Estado: Publicado, 12 de junio de 2020.

Alejandro Clavijo-Maldonado, Enio Ferreira, Carlos Vargas-Hernández and Fredy A. Rivera-Páez. *Canine mammary cancer: clinical implications with specific focus on the HER-2 gene*.

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Capítulo 2.

Estado: Publicado, agosto de 2020.

Alejandro Clavijo-Maldonado, Juan M. Pérez-Zapata; Enio Ferreira; Carlos Vargas-Hernández; Fredy A. Rivera-Páez. *Canine mammary gland tumors: risk factors and their epidemiological influence in Manizales-Colombia.*

Revista: Revista MVZ Córdoba. ISSN 1909-0544

Capítulo 3.

Estado: En preparación

Alejandro Clavijo-Maldonado; Enio Ferreira; Jorge E. Perez-Cárdenas; Carlos Vargas-Hernández; Fredy A. Rivera-Páez. *ELISA and FTIR: HER2 gene expression in blood serum of canines with mammary tumor*.

CAPÍTULO 1

Canine mammary cancer: clinical implications with specific focus on the HER-2 gene

Canine mammary cancer: clinical implications with specific focus on the HER-2 gene

Alejandro Clavijo-Maldonado, Enio Ferreira, Carlos Vargas-Hernández and Fredy A. Rivera-Páez*

Abstract

Canine mammary cancer (CMC) is one of the most common neoplasms in intact females in comparison to other species. Several risk factors have been identified, including breed, genetic predisposition, age, reproductive history, hormonal influence, diet, and body condition, in addition to previous lesions to the mammary gland, such as mammary atypical hyperplasia. An understanding of the genetic markers for the disease and a clinical approach are important for establishing a specific therapy that can allow adequate patient survivorship. Overexpression of the HER-2 gene in canines and humans is associated with a poor clinical prognosis, mainly short survivorship, although the clinical relationship is not clear. The incidence of HER-2 in female dogs can range from 29.7% to 38%. However, overexpression of HER-2 is not necessarily associated with malignancy processes of the mammary tissue, although it participates in cellular proliferation. Finally, canines remain one of the most important models for comparative oncology with humans due to the great similarity in the spontaneous presentation and development of cancer, and in the high homology in the amino acid sequence.

Key words: *c-erbB2; diagnostic; malignant mammary tumour; prognosis; risk factors*

Overview

The use of animal models for research on genetic human pathologies poses multiple advantages, including the greater size of laboratory specimens that facilitates their management (Lindblad-Toh et al., 2005; Uva et al.,

Alejandro CLAVIJO-MALDONADO*, Esp., (Corresponding author address: e-mail: alejandrocmvz@misena.edu.co), Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales, Colombia, Grupo de Propiedades Ópticas de Materiales, Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Colombia; Enio FERREIRA, PhD, Instituto de Ciências Biológicas, Departamento de Patologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil; Carlos VARGAS-HERNÁNDEZ, PhD, Grupo de Propiedades Ópticas de Materiales, Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Colombia; Fredy A. RIVERA-PÁEZ, PhD, Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales, Colombia

2009), highly similar tissue development and architecture, and the involvement of similar genes. The latter enables us to identify and understand the clinical, pathological, and genetic mechanisms involved in the onset and development of cancer (expression). Studies using canines represent an ideal solution to reduce the gap between animal models with naturally developed diseases and facilitate extrapolations to human medicine (Rowell et al., 2011).

Mammary cancer (MC) is a highly complex disease due to its heterogeneous histopathology, biological behaviour, and responses to systemic interventions (Geleick et al., 1990; Viale, 2012). Furthermore, it is considered a worldwide public health issue (Uribe et al., 2013). Canine mammary cancer (CMC) is one of the most important pathologies in veterinary medicine, particularly in non-spayed female dogs (Schneider et al., 1969; Sleeckx et al., 2011; Beck et al., 2013). Given the multifactorial origin of the disease, prevention and treatment is complex (Perez-Alenza et al., 2000). CMC is among the main causes of mortality in females and this tumour pathology has the greatest incidence compared to other domestic species (Cruz, 1999). CMC corresponds to almost 50% of all canine tumours (Misdorp et al., 1999; Dhami et al., 2010), with an incidence estimated to be three times higher than in women (Kumaraguruparan et al., 2006; Dhami et al., 2010). In addition, 50% to 70% of canine mammary tumours (CMT) are considered malignant (Moe, 2001; Merlo et al., 2008; Salas et al., 2015).

Canines as a model for naturally occurring human breast cancer (BC)

Canines are the most common pet worldwide, and it is estimated that 33% people over 15 years old own a dog. This is the most common pet in Latin America, Asia and Oceania, and second most common in Europe (GfK, 2016). This is due in part to ongoing social changes in recent years, which have led to the role of pets as "family members" (Sharpe, 2017), even influencing the owners' finances (Pet Census, 2016). As a consequence, companion animals, particularly canines, are an excellent model for studying complex human diseases (Rowell et al., 2011).

At the genome level, canines share ~650 Megabases (Mb) of ancestral sequence with humans (Lindblad-Toh et al., 2005; Pang and Argyle 2009). Also, many uni- or multifactorial physiological disorders constitute unique models for human diseases (Yang et al., 1999; Starkey et al., 2005). Nearly 400 hereditary diseases in canines have an equivalent in humans, many of which have been described and named in the same manner (Starkey et al., 2005). Therefore, as with murine models, there is increasing interest in undertaking comparative oncology research with canines (MacEwen, 1990; Pinho et al., 2012). In canines, the evolution period of cancer is significantly less than in humans, as is the response to treatment, enabling preclinical studies in cancer development (Uva et al., 2009; Peruzzi et al., 2010). As such, the time period for evaluating cancer treatment success in canines is 18 months, while at least seven years are required in humans (Paoloni and Khanna, 2008).

Canines can develop MC spontaneously (Macewen et al., 1982; Jaillardon et al., 2015) or it can be hereditary, though there is insufficient evidence despite the evident homologies with humans (Szabo et al., 1996; Goebel and Merner, 2017). The underlying genetic complexity of tumour development (Aguirre-Hernández et al., 2009), biological behaviour, growth patterns, morphology, tumour progression, metastasis patterns, histological

types, and therapeutic response are highly similar in humans (Starkey et al., 2005; Paoloni and Khanna, 2008; Pang and Argyle, 2009; Tamburini et al., 2009; Peruzzi et al., 2010; Tang et al., 2010; Gupta et al., 2012). The epidemiological characteristics are also similar between the two species (Cassali, 2013; Vascellari et al., 2016), although for some cancer types, disease progression is more aggressive in canines than in humans (Meirelles, 2010; García, 2013). Therefore, the establishment of oncology research protocols for the early detection of cancer in canines will enable its future extrapolations, followed by advantages for medicine and for human and animal survivorship.

CMC risk factors and pathogenesis

Different factors influence CMC development, including breed and genetic predisposition, age, reproductive history, hormonal activity, diet, and obesity (Sleeckx et al., 2011). Previous lesions, such as mammary atypical hyperplasia, can also increase the risk of CMC presentation (Dupont et al., 1993; Page et al., 2003; Ferreira et al., 2014).

Breed and genetic predisposition: Purebreds show a higher predisposition (Hemanth et al., 2015; Sahabi et al., 2015). French Poodle, English Springer Spaniel, English Spaniels, Cocker Spaniels, German Shepherds, Maltese, Yorkshire Terrier, and Dachshunds display a high incidence of CMC (Borge et al., 2011; Sleeckx et al., 2011; Caicedo et al., 2012; Burrai et al., 2015; Campos et al., 2015). Dhami et al. (2010) and Hemanth et al. (2015) reported other breeds that are highly susceptible to CMC development, including the Doberman, Labrador Retriever, Great Dane, Pomeranian, and Spitz (Dhami et al., 2010; Hemanth et al., 2015). Meanwhile, other breeds have been identified as low risk: Border Collie, Shetland Sheepdog, Bernese Mountain, and Saint Bernard (Borge et al., 2011). However, these results could be biased by the ownership popularity of certain breeds compared to others (Vidales and Eslava 2007; Dhami et al., 2010). Yet, there is a consensus regarding a higher cancer predisposition of small breeds compared to large breeds (Chang et al., 2005; Hsu et al., 2009; Sahabi et al., 2015).

Age: In addition to genetic predisposition, age also plays a fundamental role. CMC presentation is more frequent in mid to older age females, and an average age of six years has been defined as the "cancer age" (Perez-Alenza et al., 2000; Dhami et al., 2010; Shinoda et al., 2014). In addition, a high incidence is also observed between 9 and 10 years (Støvring et al., 1997; Hsu et al., 2009; Caicedo et al., 2012; Campos et al., 2015., Sahabi et al., 2015). However, there are also reports of age averages under 8.4 years (Chang et al., 2005), while authors such as Hemanth et al. (2015) found a higher age range of 6 to 10 years, with a reduction in frequency after 12 years.

Reproductive history: Non-spayed females are more susceptible to CMC than spayed females (Chang et al., 2005; Sleeckx et al., 2011; Hemanth et al., 2015; Sahabi et al., 2015). Females spayed before their first oestrous cycle have approximately a 0.5% risk of developing CMC, while ovariohysterectomy after the second cycle increases the risk to 8%, and to 26% after three cycles (Schneider et al., 1969). Therefore, ovarian hormone ablation through ovariohysterectomy performed during early life dramatically decreases dose-dependent steroid exposure, thus reducing the risk of early mammary tumour development (Sorenmo et al., 2011). Additionally, ovariohysterectomy increases the survival index when established as a therapeutic measure for CMC (Hsu et al., 2009), since the survival index of females with CMC that undergo ovariohysterectomy has been found to

nearly double compared to non-spayed females (Chang et al., 2005). Nevertheless, there are differences among cancer types regarding the therapeutic impact of ovariohysterectomy, e.g. in females with complex carcinoma compared to simple (Chang et al., 2005).

Hormone influence: Ovarian steroid hormones and products with medroxyprogesterone acetate (MPA) produce a proliferative effect on the mammary tissue, thus stimulating growth and increasing the risk of mammary tumour development (Sleeckx et al., 2011). Steroid hormones (mainly oestrogens and progesterone) participate in the normal development of the mammary tissue and play a key role in the early stages of CMC pathogenesis (Sorenmo et al., 2011). Therefore, an ovariohysterectomy after the second oestrous cycle does not guarantee full protection from possible tumour development (Schneider et al., 1969). This can be explained by the fact that both oestrogen receptors (ER) and progesterone receptors (PR) are present in high amounts in normal tissues and benign lesions, leading to accumulation and availability in the mammary tissue (Macewen et al., 1982; Rutteman et al., 1988; Clamp et al., 2003; Rao, 2008), as compared to cancerous or metastatic tissues. Nevertheless, there is a notable presence of 17β-[^{3}H] oestradiol (Macewen et al., 1982). Furthermore, MPA, a progestin used to prevent oestrous or treat false pregnancy, is known to increase the risk of CMC development (Rutteman et al., 1988; Støvring et al., 1988). MPA induces overproduction of the growth hormone (GH), which induces the development of dysplasia and benign tumours (Perez-Alenza et al., 2000). The interaction of the GH in the mammary tissue stimulates the insulin-like growth factor 1 (IGF-1).

Prolactin (PRL) is a 199 amino acid peptide with a molecular weight of 23 kDa (Freeman et al., 2000), synthesized by the anterior pituitary gland. This peptide displays a luteotrophic action that is especially important during the second half of pregnancy (Verstegen-Onclin and Verstegen, 2008; Rufo et al., 2016), participates in cellular development and differentiation of the canine mammary gland (Jöchle, 1997; Rufo et al., 2016), and carries out lactogenic activity (Michel et al., 2012a), among other functions. Although the role of PRL in tumour development in the canine mammary gland is still under debate, several studies have proposed that tumour genesis in the pituitary gland is associated with decreased secretory activity (El Etreby et al., 1980). This agrees with later studies that reported a reduced expression of the prolactin receptor (PRLR) (Michel et al., 2012b). In addition, findings have shown high levels of PRL in CMC compared to benign lesions or hyperplasias (Queiroga et al., 2005). These authors indicate that most PRL present in cancerous tissues is not of pituitary origin. This has been demonstrated in humans (Ginsburg and Vonderhaar, 1995), where there might be an autocrine and paracrine effect (Ben-Jonathan et al., 2002; Clevenger et al., 2003).

Diet: Homemade food, such as those rich in fat and beef and pork, increase susceptibility to CMC compared to diets rich in poultry or balanced diets (Alenza et al., 2000; Sleeckx et al., 2011).

Overweight and obesity: Body fat ranges from 15 to 25% in healthy animals, while more than 30% is considered obesity (Burkholder et al., 2000). However, this relationship tends to be narrower as age increases (German, 2006). Body mass and overweight status can be assessed through morphological analysis with determination of body fat (Burkholder et al., 2000), e.g. the Canine Body Mass Index (IMCC) (Muller et al., 2008) or Escore of Body Condition (ECC) (Laflamme, 1997). Adipocytes are

the functional unit of fatty tissue (Khan et al., 2015). They have high metabolic activity and are highly sensitive to nervous, nutritional and hormonal control (Stephens, 2012) and about 95% of the cell weight is represented by triglycerides (Khan et al., 2015). However, being overweight alters the release of certain substances, including leptin (German et al., 2010). This protein hormone specific to adipocytes of 167 amino acids, has been known for its appetite control effects (Facey et al., 2017) and is linked to the regulation of body mass (Hassink et al., 1996).

In dogs and humans, an increase in leptin has been found when adipose tissue is more abundant (Maffei et al., 1995; Gayet et al., 2004; Kil and Swanson, 2010), implying cell proliferation by stimulation of the IGF-1 or somatomedin and promotion of angiogenesis (Renehan et al., 2006). In BC, the leptin receptor (ObR) and Human Epidermal Growth Factor Receptor type 2 (HER-2) can be co-expressed, which reduces the effectiveness of HER-2 treatments (Fiorio et al., 2008), since ObR can mediate HER transactivation-2 (Soma et al., 2008), although this is controversial (Santillán et al., 2012). The positive relationship between ObR and HER-2 has also been observed in animals (rodent models) with mammary tumour (García-Robles et al., 2013), although this interaction is not yet clear (Lim et al., 2015).

In female dogs, obesity during the first year of life markedly increases the risk of cancer, since several carcinogenic events occur in the mammary gland during this period, although there is no clarity on this mechanism (Sonnenschein et al., 1991; Perez-Alenza et al., 2000; Sorenmo et al., 2011; Lim et al., 2015). Several findings have shown that the risk is similar if a female is obese at least one year prior to being diagnosed with CMC (Shofer et al., 1989).

Clinical aspects and diagnosis of CMC

Clinically, tumour masses are the main warning of abnormality in the mammary glands of female dogs. The size of these masses can range from 0.5 cm to 21 cm in diameter (Chang et al., 2005; Hsu et al., 2009). Consequently, the clinical significance of canine tumour masses indicates that small and hard masses are more likely benign, while larger masses generally display ulceration and are histologically malignant (Hemanth et al., 2015). Thus, the latter result in a more unfavourable prognosis (Hsu et al., 2009).

All mammary glands can be involved in the development of CMC, whether initially one gland is involved or a combination of them, and these can show the same or different histological features (Perez-Alenza et al., 2000; Goebel and Merner, 2017). There is no tendency towards one side more than the other (Chang et al., 2005; Hsu et al., 2009; Hemanth et al., 2015), though findings have shown that the caudal mammary glands (glands 3, 4 and 5) are more affected than the thoracic pairs (1 and 2) (Chang et al., 2005; Hsu et al., 2009). In consequence, the inguinal zone is most affected, while the least affected is the caudal thoracic sector (Hemanth et al., 2015).

Additionally, lymphatic drainage in healthy females is ipsilateral (towards the same side) and there is no evidence of drainage towards the contralateral lymph nodes (LN) (Pereira et al., 2003; Pereira et al., 2008), which is one of the differentiating factors in neoplastic glands (Patsikas et al., 2006). However, not all CMC types behave in the same manner, e.g. epithelial type neoplasms, such as carcinomas, generate metastasis through the lymphatic system, while mesenchymal neoplasms, such as sarcomas, achieve metastasis through capillaries and veins (Sorenmo et al.,

2011). There are correlations between CMC patient survival and the number of affected LN, where average survivorship decreases with increasing number of affected LN (Carter et al., 1989; De Araújo et al., 2015). Tumour size and LN condition are independent prognostic factors; however, they are additive (Carter et al., 1989), since a larger tumour diameter is related to metastasis to the lymph node (Chang et al., 2005). This fact indicates a direct relation between these two factors.

CMC and genetic markers

In hereditary human BC, alterations of the suppressor gene p53 and mutations in suppressor genes BRCA1 and BRCA2 (Breast Cancer 1, 2) are mainly involved (Overgaard et al., 2000; Honrado et al., 2006; Olivier et al., 2006; Pérez-Losada et al., 2011). In sporadic MC, there is an involvement of modulators of cellular proliferation, including alterations in ER, which are classified into three groups: classic ERα, ERβ, and the most recently described, GPR30 (G protein-coupled receptor 30) (Prossnitz et al., 2007; Hazell et al., 2009; Prossnitz and Maggiolini, 2009). The first two ER groups belong to the superfamily of nuclear receptors that regulate elements at the nuclear level through MAPK-type responses (*Mitogen-Activated Protein Kinases*), PI3K (*Phosphoinositide (PI) 3-Kinase*), and cAMP (*cyclic adenosine monophosphate*). Alterations in these two receptors lead to cellular proliferation, growth, and survival. ER are implied in high resistance to treatments and development of metastasis, in co-expression with the HER-2 gene (Filardo et al., 2006). Another group consists of *Transforming growth factor* (TGF β), which includes three members (TGF β I-III). A reduction in the expression of TGF β enables the development of MC and eventual metastasis (Landis et al., 2005; Dong et al., 2007). In addition, PGDF (Platelet-Derived Growth Factor) receptors include two types, PGDFR α and β, which are related to cellular proliferation and differentiation. Tumour invasion capacity has been correlated with co-expression of PGDFR α and HER-2 (Carvalho et al., 2005). Finally, the Protease-activated receptor (PAR) participates in the modulation of cancer growth (Ceballos and Hernández, 2008). PAR1 mediates calcium signalling, transcription processes, and mitogenesis (Coughlin, 2000). HER-2 contributes to PAR1 activation, therefore, providing high prognostic value (Ceballos and Hernández, 2008).

In canines, mutations in genes with high or low penetrance in cancer considerably increase the risk of CMC presentation. Single Nucleotide Polymorphisms (SNP) in coding regions can lead to alterations in protein structure or function (Borge et al., 2011). Genes that show different risk levels have been the most studied, including breast cancer susceptibility genes 1 and 2 (BRCA1, BRCA2), tumour protein p53 (TP53), phosphatase and tensin homolog (PTEN), checkpoint kinase 2 (CHEK2), ataxia telangiectasia mutated (ATM), and human epidermal growth factor receptor-2 (HER-2) (Hsu et al., 2009; Borge et al., 2011).

CMC and clinical implications of the HER-2 gene

In women with BC, the overexpression of HER-2 (HER-2 positive state) is found between 20% and 30%, and is generally correlated with a high phenotypic aggressiveness and resistance to cytotoxic and endocrine therapies (González et al., 2007; Fehm et al., 2007; Savino et al., 2009; Park et al., 2014). The latter leads to a poor clinical prognosis, mainly short survivorship (Slamon et al., 1987; Gambini et al., 2003; Ross et al., 2003),

and in both humans and canines, the prognosis is reserved for up to two years after surgery (Ressel et al., 2013).

The oncogene HER-2/*neu* was initially isolated from neuroectodermal tumours in rats and compared to its homologues in humans and rabbits; therefore, *neu* corresponds to neuroblastoma (Shih et al., 1981). In humans, HER-2/*neu* maps to chromosome 17q 12-21.32 (Akiyama et al., 1986; Fukushige et al., 1986; Popescu et al., 1989; Fehm et al., 2007; Finn et al., 2009; Krishnamurti and Silverman, 2014). In canines, the gene is located on chromosomes 9 and 5 (Yang et al., 1999); however, it has also mapped to chromosome 1q13.1 through fluorescence *in situ* hybridization (FISH) (Murua Escobar et al., 2001). HER-2/*neu* is a membrane protein of 185-kDa (Manguire et al., 1989), conformed by three domains: a transmembrane lipophilic domain, an extracellular domain (ECD) (105 -kDa), and an intracellular tyrosine kinase domain (Ha et al., 2015; Di Gioia et al., 2015). HER-2 carries out an important role in regulating cellular growth and differentiation (Yarden, 2001). However, it has gained importance given its participation in the physiopathological progression of the mammary tumour and low response to treatments (Akiyama et al., 1986; Lüftner et al., 2003).

In humans, the clinical significance of HER-2 is relevant, because it is overexpressed in early stages of cancer development. In consequence, it has become a therapeutic target (Hanna, 2001; Yarden, 2001; Wilson et al., 2002; Reddy et al., 2004; Finn et al., 2009; Krawczyk et al., 2009; Onitilo et al., 2009; Page et al., 2011; Soares et al., 2016). Yet, unlike the other receptors (HER-1, HER-3, and HER-4), HER-2 oncogenesis is attributed to an increase in the expression of a non-mutated receptor. As a result, there is an increase in tyrosine-kinase activity, which induces cellular transformation (Siegel et al., 1994; Biscardiet et al., 2000; Yarden 2001; Stefano et al., 2004; Moasser, 2007). Therefore, HER-2 is closely related with the rate of cancer progression (González et al., 2007). This is due to a deletion in exon 16 of the extracellular domain (Siegel et al., 1994) and a polymorphism in codon 655 (Papewalis et al., 1991). Hence, MC with overexpression of HER-2 highly correlates with metastasis in regional lymph nodes and, for this reason, HER-2 is used as a prognosis marker in relation to other proteins p53, Ki67, ER, and PR (Selvarajan et al., 2004).

There is a 29.7% incidence of HER-2 overexpression in female canines diagnosed with malignant mammary tumour (Hsu et al., 2009). Later studies showed incidence rates of 28.6% (Ressel et al., 2013), 38% (Shinoda et al., 2014), 37.5% (Burrai et al., 2015) and 32.1% (Campos et al., 2015). However, the results regarding HER-2 expression levels are not yet clear (Hsu et al., 2009; Ressel et al., 2013). HER-2 overexpression is seemingly not strictly associated with the initial stages of atypical cellular proliferation (Ferreira et al., 2014), indicating a high complexity in terms of establishing the most adequate prognosis and therapy for each type of CMC (Resssel et al., 2013).

In canines, it is likely that HER-2 only participates in proliferation and not in the malignancy process of the mammary tissue during tumour formation (Hsu et al., 2009; Ressel et al., 2013). Nevertheless, this can vary, since there have been reports of CMC processes with amplification or overexpression of HER-2 (Rungsipipat et al., 1999), and of others with no association to HER-2 (De las Mulas et al., 2003). This poses a challenge for prognosis (Dutra et al., 2004), as findings are not clear regarding the effects of HER-2 on the survivorship rate in comparison to other genes (Hsu et al., 2009; Shinoda et al., 2014). However, a HER-2 positive state means an unfavourable prognosis, both for women (Savino et al., 2009) and

in most canine female cases (Hsu et al., 2009; Ressel et al., 2013).

HER-2 as a tumour marker in CMC

A tumour marker (TM) is a molecule (glycoprotein, generally) that can be produced by normal cells and tumour cells, although levels are higher in the presence of cancer (Hermida et al., 2016). In cancer, concentrations of TM can be produced by both normal cells and cancer cells, and these substances are detectable in biological fluids (Romero et al., 2002; Almeida et al., 2007). TMs may be tumour-specific proteins (tumour antigen specific), nonspecific protein tumour markers associated with malignant cells, or specific proteins overexpressed in malignant cells (Lindblom and Liljegren, 2000; Romero et al., 2002). No TM is totally sensitive and specific, but together with other clinical procedures can take specific therapeutic decisions (Koshida et al., 1996; Lindblom and Liljegren, 2000; Hermida et al., 2016).

HER-2/*neu* (HER-2) is a proto-oncogene that encodes a glycoprotein that stimulates cell proliferation and differentiation in normal epithelial cells (Yarden and Sliwkowski, 2001; Farzadnia et al., 2010). In humans, overexpression of the HER-2 protein has been found in 20 to 30% of invasive breast cancer cases (Slamon et al., 1987; Wolff et al., 2013). The concentration in serum has an important prognostic value (Andrulis et al., 1998; Sjögren et al., 1998; Agrup et al., 2000) and it has high influence on treatment decisions (Leyland-Jones, 2002).

In human breast cancer (HBC), HER-2 levels in blood serum have been studied in metastatic cancer (Jensen et al., 2003). Serum concentrations of 18.5% have been reported (Carney et al., 2003), and 13.4% with significant correlation with tumour size and clinical grade by immunohistochemical analysis (IHC) (Harris et al., 2001) and ELISA in primary HBC (Pallud et al., 2005). Other studies have not found a positive correlation between clinical-pathological variables and elevated serum levels (Kong et al., 2006). The cut-off value for serum measurements of HER-2 should be determined for each type of population (Ellis et al., 2000; Rakha et al., 2015) due to the little relation that there could be with the amplification of the gene or the overexpression of the protein (Kong et al., 2006).

The BLAST alignment of HER-2 reveals a 92% homology in the amino acid sequence between humans and canines (Singer et al., 2012). However, the use of human test kits for enzyme-linked immunosorbent (ELISA) to measure concentrations of HER-2 in dogs did not significantly differentiate between healthy patients and those with cancer (Campos et al., 2015). However, the correlation of serum levels of HER-2 with tumour size, high histological grade, mitotic index and nuclear polymorphism indicated a poor prognosis (Dutra et al., 2004; Hsu et al., 2009; Muhammadnejad et al., 2012; Kaszak et al., 2018), although this relationship is controversial (Kim et al., 2011; Ressel et al., 2013). Additionally, reduced expression has been reported in the presence of aggressive tumours such as ducal carcinoma *in situ* (DCIS) (Silva et al., 2014), unlike that found in feline mammary cancer (FMC) (Soares et al., 2016).

Currently, it has been difficult to determine the similarities or differences in the overexpression of HER-2 between humans and canines. In canines, we find the deletion of exon 16 or the absence of polymorphism of codon 655, yet there is a polymorphism in exon 14 (Hsu et al., 2009). Nonetheless, it is important to differentiate between diagnostic methods with high sensitivity, in order to clearly establish similarities and relations in

this gene between humans and canines (Savino et al., 2009).

Conclusions

The differences between the overexpression of HER-2 in human BC and CMT are not yet clear. Despite recognizing the importance of HER-2, the clinical usefulness of its detection in animal medicine is still unclear, since the relationship between overexpression of HER-2 and CMC may be due to the interaction of several genes and not to the activity of the gene itself. In addition, care must be taken to determine HER-2, since it is overexpressed in other types of tumours, therefore it is necessary to supplement the clinical correlation with other clinical analyses. In humans, high levels of ECD in primary BC have a high diagnostic, prognostic and therapeutic value, while in canines, the pattern of presentation is not clear and survival findings are controversial. Given the high complexity involved in monitoring HER-2 in female dogs, it is important to develop studies with simple, minimally invasive methods that would allow for early detection.

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Tumor mliječne žlijezde kuja: kliničke implikacije sa specifičnim fokusom na HER-2 genu

Alejandro CLAVIJO-MALDONADO, Esp., Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales, Colombia, Grupo de Propiedades Ópticas de Materiales, Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Colombia; Enio FERREIRA, PhD, Instituto de Ciências Biológicas, Departamento de Patologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil; Carlos VARGAS-HERNÁNDEZ, PhD, Grupo de Propiedades Ópticas de Materiales, Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Colombia; Fredy A. RIVERA-PÁEZ, PhD, Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Manizales, Colombia

Tumor mliječne žlijezde kuja je jedna od najčešćih neoplazija u ženki u usporedbi s drugim vrstama. Ustvrđeno je nekoliko čimbenika rizika uključujući pasminu, genetsku predispoziciju, dob, reproduktivnu anamnezu, hormonalni utjecaj, hranidbu i tjelesnu kondiciju uz prethodne lezije mliječne žlijezde kao što su primjerice atipična hiperplazija mliječne žlijezde. U cilju uspostavljanja specifične terapije koja bi omogućila prihvatljivo vrijeme preživljavanja pacijenata važno je razumijevanje genetskih markera za spomenutu bolest kao i klinički pristup. Prekomjerna ekspresija HER-2 gena u kanida i ljudi povezana je s nepovoljnom kliničkom prognozom, uglavnom s kratkim vremenom preživljavanja, premda nije jasna njihova klinička povezanost. Incidencija HER-2 u kuja može biti u rasponu od 29,7 % do 38 %. Međutim, prekomjerna ekspresija HER-2 nije nužno povezana sa zloćudnim procesima u tkivu mliječne žlijezde, premda ima ulogu u staničnoj proliferaciji. Naposljetku, kanidi su i dalje najvažniji modeli za komparativnu onkologiju u odnosu na ljude zbog velike sličnosti u spontanom izgledu i razvoju tumora kao i u visokoj homologiji u slijedu aminokiselina.

Ključne riječi: *c-erbB2, dijagnostika, zloćudni tumor mliječne žlijezde, prognoza, čimbenici rizika*

CAPÍTULO 2

Canine mammary gland tumors: risk factors and their epidemiological influence in Manizales-Colombia

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Original

Canine mammary gland tumors: risk factors and their epidemiological influence in Manizales-Colombia

Alejandro Clavijo-Maldonado[1,4*] Esp; Juan M. Pérez-Zapata[2] M.Sc; Enio Ferreira[3] Ph.D; Carlos Vargas-Hernandez[4] Ph.D; Fredy A. Rivera-Páez[1] Ph.D.

[1]Universidad de Caldas, Facultad de Ciencias Exactas y Naturales, Departamento de Ciencias Biológicas, Grupo de Investigación GEBIOME, Manizales, Colombia.
[2]Universidad de Caldas, Facultad de Ciencias Agropecuarias, Departamento de Salud Animal, Manizales, Colombia.
[3]Universidade Federal de Minas Gerais Departamento de Patologia, Geral, Instituto de Ciências Biológicas, Belo Horizonte, Minas Gerais, Brasil.
[4]Universidad Nacional de Colombia, Departamento de Física y Química, Propiedades Ópticas de Materiales, Manizales, Colombia.
*Correspondence: alejandrocmvz@misena.edu.co

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ABSTRACT

Objective. To describe the prevalence of canine mammary gland tumors (CMT) in females in the municipality of Manizales-Colombia from 2014-2017. **Materials and methods.** A database of 15961 patients was consolidated. The variables analyzed were, breed, age, reproductive history, weight, diet type and clinical characterization of the tumor with the TNM (tumor-node-metastases) staging system. The statistical analysis include Pearson's chi-squared test with Yates correction for continuity and Fisher's exact test ($p<0.05$), relative risk and odds ratio (CI95%) and Kaplan-Meier estimator for survival analysis. **Results.** The incidence in purebred dogs was 79.14%, with a peak at the average age of 9.3 years old. Pearson's chi-squared test and the relative risk and odds ratios indicated a high risk for purebreds ($p=0.019$, 3.96/100, 1.64, respectively). Females of ages between 9 and 12 years old showed a 74% likelihood of developing a mammary tumor. No found significant relation to weight or reproductive stage but indeed a high association with homemade diet ($p<0.001$). The inguinal mammary pairs were the most affected (6.9%). The Kaplan-Meier estimate showed a higher survival of surgically-intervened patients, with 2013 days of survival after diagnosis with surgery compared to 1484 days without surgery. **Conclusions.** The study confirmed the relevance of risk factors, breed type, age, body condition and diet type in the mammary tumor presentation. Furthermore, it highlights the need for improving and integrating the veterinary diagnostic information systems, considering their importance in public health.

Keywords: Cancer; epidemiology; incidence; prevalence (*Source: CAB*).

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RESUMEN

Objetivo. Describir la prevalencia del tumor mamario canino (TMC) en hembras en el municipio de Manizales-Colombia durante 2014-2017. **Materiales y métodos.** Se consolidó una base de datos de 15961 pacientes. Las variables analizadas fueron raza, edad, historia reproductiva, condición corporal, tipo de dieta, y caracterización clínica del tumor mediante el sistema de estadificación tumor-nódulo-metástasis (TNM). El análisis estadístico incluyó prueba de chi-cuadrado de Pearson con corrección de Yates, prueba exacta de Fisher ($p<0.05$), riesgo relativo, oportunidad relativa (IC95%) y análisis de supervivencia de Kaplan-Meier. **Resultados.** La incidencia en perros de razas puras fue 79.14%, con un pico promedio a la edad de 9.3 años. La prueba chi-cuadrado de Pearson, el riesgo y las oportunidades relativos mostraron un riesgo alto para razas puras ($p=0.019$, 3.96/100, 1.64, respectivamente). Hembras entre los 9 y 12 años mostraron una probabilidad del 74% de desarrollo de tumor mamario. No se encontró una relación significativa entre la masa corporal o estado reproductivo pero una elevada asociación con la dita casera ($p<0.001$). Los pares inguinales mamarios fueron los más afectados (6.9%). La estimación de Kaplan-Meier mostró una alta supervivencia para pacientes intervenidas quirúrgicamente, con 2013 días después del diagnóstico con cirugía en comparación con 1484 días sin cirugía. **Conclusiones.** El estudio confirma la relevancia de los factores de riesgo: tipo de raza, edad, condición corporal y tipo de dieta en la presentación de tumor mamario. Además, se destaca la necesidad de mejorar e integrar los sistemas de información de diagnóstico veterinario, considerando su importancia en la salud pública.

Palabras clave: Cáncer; epidemiologia; incidencia; prevalencia (*Fuente: CAB*).

INTRODUCTION

Canine mammary gland tumor (CMT) is one of the most common tumors in dogs (1) and one of the main causes of mortality, similarly, as occur in humans (2,3). In female dogs, 40% of tumors are of the mammary gland (4,5) and these are three times more frequent than in women (1,6). In particular, intact females are more susceptible (4,7,8,9,10,11).

Canine mammary tumors are the second most frequent type after skin tumors (12,13). There are several predisposing factors to the presentation of CMT. For instance, purebreds are more susceptible (9,10) and, among these, small breeds are more susceptible to CMT (7,10,14). Age-wise, the highest incidence of CMT is estimated from 8 to 10 years old (1,8,9,10,13,14,15,16).

Early sterilization greatly reduces the susceptibility of developing CMT (17) and increases the survival rate (7,14). Ovarian steroid hormones or exogenous products, such as medroxyprogesterone acetate, stimulate the proliferation of the mammary tissue and, consequently, increase the risk of CMT (8,17,18). Another relevant risk factor is excess weight and obesity, which markedly increase the risk of CMT (19). Although other authors have not found this association (20). Homemade diets rich in fat is another risk factor (8,19). Many of these risk factors are shared with humans, in particular, lifestyle, type of diet/obesity, and hormonal birth control therapies (21,22).

Although CMT is a relatively common disease, in several places the databases are not consolidated, represents a challenge for data retrieval (23). Many clinical records are incomplete, ambiguous, or do not indicate the definitive diagnosis or the length of survival of the patients (24). The main objective of this study was to describe the prevalence of CMT in females and males in the municipality of Manizales-Colombia from 2014-2017.

MATERIALS AND METHODS

Data retrieval. We consolidated a database from clinical records between 2014 and 2017 provided by eight veterinary care centers in the city of Manizales (Caldas, Colombia). Access to the clinical records was done with signed consent from the animal owners and/or veterinarians. The constructed database was filtered to establish four groups of patients: Group I: all patients reported from consultations. Group II: patients from group I with a clinical diagnosis. Group III: patients from group II with any type of tumor affection. Group IV: it includes patients

from group III with a mammary gland tumor. The variables analyzed in the females diagnosed with CMT were breed (25), and breed type (purebred or mixed-breed), age, weight, type of diet, reproductive stage, hormonal treatments, and clinical characterization of the tumor (type, size, number of affected mammary glands, affected location, and metastasis).

We estimated the rate of presentation of tumor pathologies each year and determined the most affected organ system, as well as the annual number of females diagnosed with CMT. The topographic classification was done based on the Classification of Disease for Oncology System (ICD-O) (26). We determined the disease prevalence according to breed, age, and weight (normal, overweight, or obese based on the breed). In dogs, overweight is considered being 15% more than the "optimal weight" (27,28) and obese when it exceeds 30% (29).

The clinical characterization of the tumors was determined based on the TNM staging system (30). The histopathological findings were adapted according to Cassali et al (31). The data were consolidated in a MS Excel® spreadsheet. We also searched the bank of histopathological slides and histopathological reports databases of the consulted veterinary attention centers. We made a photographic register with a Leica ICC50 HD camera system and analyzed the images with ImageJ software (Wayne Rasband, National Institutes of Health, USA). Finally, we confirmed by telephone, the survival time from the initial diagnosis and surgical procedure of the females.

Statistical analysis. The relationship between the variables breed, age, weight, type of diet, and reproductive stage was determined using Pearson's chi-squared test with Yates correction for continuity and Fisher's exact test for continuous data with $p<0.05$. We also estimated epidemiological indices including incidence rate (relative risk – RR) for the female population over six years old with a definitive diagnosis and odds ratio – OR with a confidence interval of 95% (CI95%). The RR was calculated as the total number of canine females per 100 females-years divided by the total number of diagnosed females. The OR was estimated using 2x2 tables for each the study variables. The data was consolidated in a MS Excel® spreadsheet and analyzed using the R statistical package v.3.5.3 (R Foundation for Statistical Computing, Vienna, Austria). The survival analysis was performed using the Kaplan-Meier estimator ($p<0.05$) with GraphPad Prism v.8.2.0 software (GraphPad Software Inc., San Diego, CA).

RESULTS

General prevalence. The consolidated database (2014-2017) allowed distributing the patients into four groups. Group I (n=20815); Group II (n=15961), 15258 canines; Group III, tumor alterations (n=403), female and male canines; Group IV, females with CMT diagnosis (n=139) (Table 1). We classified the data into 30 categories for each tumor alteration (ICD-O), in addition to a non-determined category. The general prevalence of the tumor alterations was 2.52% (403/15961). The most affected system during 2014-2017 was the mammary gland (code C50), 34.5%, followed by the skin (C44), 88 cases (21.8%). We found 139 alterations of the mammary gland that corresponded to CMT. We excluded those of different origin. The general prevalence of CMT was 0.87% (139/15961).

Prevalence by breed, age, and body condition. Purebreds were more affected (79.14%) compared to mixed-breeds (20.86%) (Table 2). The most affected breeds were French Poodle (24%), Pinscher (10%), Miniature Schnauzer, Cocker Spaniel, Beagle, and Labrador Retriever (Table 2). The mean age of the patients was 9.3 years old (SD= ±3.68) and the median was 10 years. The age range was Xmin 1year - Xmax 16 years. The most reported age ranges were 10 to 12 years old (n=24; 17.3%), followed by 7 to 9 years (n=21; 15.1%). Females older than 12 years (n=13; 9.4%), 4 to 6 years old (n=7; 5%) and less than 4 years old (n=6; 4.3%). We found that 48.9% (n=68) of the clinical records did not describe the age of the patient. Moreover, 75.5% (n=80) were patients with normal weight, 10.4% (n=11) were overweight, and 15 patients were obese (n=15; 14.1%). We did not include mixed-breeds (n=29).

Pearson's chi-squared test with Yates' correction for continuity showed a chi-squared value of 5.4287 ($p=0.019$) for breeds, indicating a positive relationship between breed and CMT. This result agrees with the breed-wise RR of 3.96/100-females, indicating a high risk (OR=1.64). Fisher's test showed a strong relationship between CMT and age ($p<0.001$). The ages from 9 to 12 years showed the highest significant risk for CMT (OR 2.81). We did not find a significant relationship between body mass (weight) and the presence of CMT ($p=0.051$).

Table 1. Tumor alterations by organ system in canines of Manizales, Colombia from 2014-2017.

Sitie	Topographic Code (ICD-O)[a]	Total (%)		Female (%)		Male (%)	
Tongue, UP	C02	1	0.2	1	0.2		0
Mouth floor	C04	1	0.2		0	1	0.2
Mouth UP	C06	18	4.0	8	1.8	10	2.2
Nasopharynx	C11	2	0.4	1	0.2	1	0.2
Small intestine	C17	1	0.2	1	0.2		0
Anus and anal canal	C21	2	0.4		0	2	0.4
Liver and intrahepatic ducts	C22	9	2.0	2	0.4	7	1.6
Other digestive organs (intestine, GI)	C26	1	0.2	1	0.2		0
Bronchi and Lung	C34	1	0.2	1	0.2		0
Heart, mediastinum, pleura	C38	1	0.2	1	0.2		0
Bones, joints, cartilage	C40	1	0.2	1	0.2		0
Bones, joints, cartilage, UP	C41	2	0.4	1	0.2	1	0.2
Hematopoietic and reticuloendothelial system	C42	3	0.7	1	0.2	2	0.4
Spleen	C42.2[a]	8	1.8	1	0.2	7	1.6
Skin	C44	88	19.7	37	8.3	51	11.4
Connective, subcutaneous and other tissue	C49	8	1.80	6	1.3	2	0.4
Mammary gland	C50	139	40.8	139	31.4		0
Vulva	C51	1	0.2	1	0.2		0
Vagina	C52	23	5.2	23	5.2		0
Uterus	C55	1	0.2	1	0.2		
Ovaries	C56	1	0.2	1	0.2		0
Penis	C60	25	5.6		0	25	5.6
Prostate gland	C61	4	0.9		0	4	0.9
Testicles	C62	13	2.9		0	13	2.9
Kidney	C64	2	0.4	1	0.2	1	0.2
Eyes and annexes	C69	5	1.1	1	0.2	4	0.9
Brain	C71	2	0.4	1	0.2	1	0.2
Thyroid gland	C73	1	0.2		0	1	0.2
Lymph nodes	C77	1	0.2		0	1	0.2
Unknown Primary Site	C80	1	0.2		0	1	0.2
Undetermined	ND	37	8.3	26	5.8	11	2.5
Total		403	100	257	63.8	146	36.2

[a]ICD-O: Structure of the topographic code. Site=(C00), sub-site=(.0).

Table 2. Breed-wise morbidity from CMT in canines of Manizales, Colombia, according to the classification by Salt et al (25).

Breed type (Kg)	Breed	Total cases	Proportion(%)
I (< 6.5)	Chihuahua	1	1
	Maltese	1	1
	Pinscher	14	10
	Yorkie	1	1
	Yorkshire Terrier	4	3
Total		21	15.1
II (6.5 to < 9)	Jack Russell Terrier	1	1
	Pekingese	1	1
	Miniature schnauzer	5	4
	Shih Tzu	2	1
Total		9	6.5
III (9 to < 15)	French bulldog	2	1
	Cocker Spaniel	5	4
	Pug	1	1
	Beagle	5	4
	Boston terrier	1	1
	Fox Terrier	1	1
	French Poodle	34	24
Total		49	35.3
IV (15 to < 30)	Basset Hound	3	2
	English bulldog	3	2
	Pit Bull	4	3
Total		10	7.2
V (30 to < 40)	Golden Retriever	4	3
	Labrador Retriever	6	4
	German shepherd	4	3
	Siberian Husky	4	3
Total		18	12.9
VI (40+)	Rottweiller	3	2
Total		3	2.2
Other	Mongrel	29	21
Total		29	20.9
Total		139	100

The chi-squared test indicated a relationship between CMT and homemade food diet ($p<0.001$) and a RR=1.8 showed a high risk for this variable. We did not find a relationship for CMT and the reproductive stage ($p=0.913$), OR=1.47, indicating it is a non-significant risk factor. Although, this could be attributed to the few reports on the reproductive stage of the patients (Table 3).

Clinical aspects. Twenty-four clinical records provide reports of TNM staging the left mammary ridge being the most compromised. The inguinal pairs are the most affected (pair 5: 18.2% left and 15.6% right). For the variable diameter of the primary tumor (T) of the TNM staging system, we found that T1 (<3cm diameter) is the most reported with 38.46%, followed by T3 (3-5cm, 29.23%), and T2 (> 5cm, 20%). T4 (inflammatory carcinoma) comprises tumors adhered to the skin (6.15%), as well as non-fixed (4.62%) and 1.54% fixed to the muscle. Absence of compromise to the regional lymph node-LN (N) and distant metastasis (M) was 70.8% (N0, M0), N1 and M1 (29.2%) for both. The predominant Clinical Stage (CS) was CS-V (25%), followed by CS-I, CS-III, and CS-IV (20.8%), respectively, and CS-II, 12.5%.

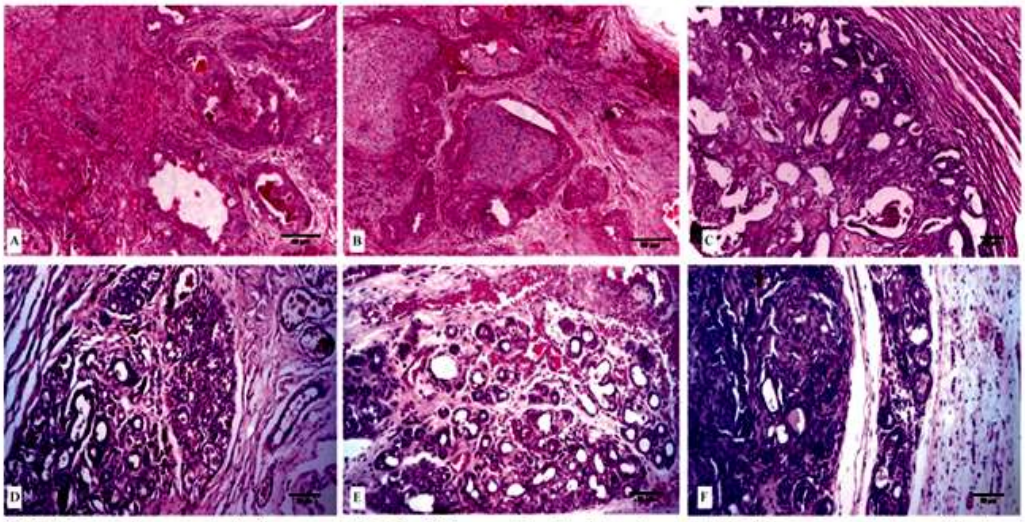
Of the histopathological findings, 18% were benign neoplasia, non-determined (ND) hyperplasia, mammary adenoma, and fibroadenoma. On the other hand, 5% were malignant neoplasia, mammary carcinoma-ND, papillary carcinoma, tubular carcinoma, and mixed tumor carcinoma. Figure 1 shows histopathological diagnostic slides of some of the females included in this study. Unfortunately, 77% were CMT reports with no definitive histopathological diagnosis. Metastases to the lung and the LN were reported in 31% and 38% of cases, respectively.

The Kaplan-Meier curve indicated a higher survival of patients that were surgically intervened, showing a survival period of 2013 days after diagnosis with surgery compared to 1484 days without surgery. The survival period after surgery was 2005 days. We did not observe significant differences for both analyses ($p=0.31$ and 0.183, respectively) (Figure 2). The analysis of the clinical records was challenging, particularly due to incomplete records and unclear reports of the clinical findings.

Table 3. Odds Ratio (OR) with a 95% confidence level (95%CI), probability and risk for patients diagnosed with CMT. Variables breed, age, weight, type of diet, reproductive stage.

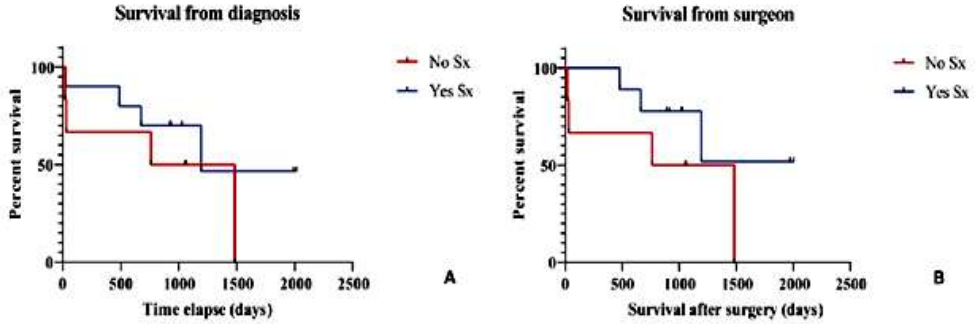
Category	CMT		Total	OR	95% CI	Probability
	Diseased	Healthy				
Breed						
Pure	103	233	336	1.64[a]	1.08-2.49	62%
Mongrel	26	73	99	0.61[b]	0.40-0.92	38%
Age (Years)						
0-4	19	167	186	0.3[b]	0.16-0.56	23%
5-8	19	167	186	0.36[b]	0.18-0.71	26%
9-12	22	80	102	2.81[b]	1.42-5.58	74%
≥ 13	19	55	74	1.33[c]	0.59-3.03	57%
Weight						
Normal	80	65	145	0.64[c]	0.39-1.03	39%
Overweight / Obesity	26	22	48	1.57[c]	0.97-2.56	61%
Feed Type						
Balanced feed	27	176	203	0.14[b]	0.08-0.26	12%
Mixed / homemade food	14	14	28	7.05[a]	3.81-13.04	88%
Reproductive state						
Intact	32	130	162	1.47[c]	0.68-3.16	60%
Spayed	7	66	73	0.68[c]	0.32-1.47	40%

Meaning: [a] Risk factor, statistically significant; [b] Protection factor, statistically significant; [c] Not significant



(A) Tubular carcinoma with *in situ* areas. H&E. 10x. Pleiomorphic epithelial cells and mitotic figures present. Loss of the basal layer continuity. (B) Mixed tumor carcinoma. H&E. 40x. (C) Tubular carcinoma. H&E. 10x. Epithelial proliferation in tubule configuration. (D). Adenosis. H&E. 10x. Periductal tissues altered and lobular dilation. (E) Adenosis with hemorrhage. H&E. 10x. (F) Benign mixed mammary tumor. H&E. 10x. Mesenchymal proliferation and high epithelial cellularity.

Figure 1. Photomicrographs of tissue after hematoxylin and eosin staining (H&E), showing histopathology of mammary lesions in canines (Manizales, Colombia). Scale bars equal 40 or 50 µm.



Sx: Surgery

Figure 2. Kaplan-Meier survival curve. Female canines with CMT. Difference in survival between groups without (A) and with (B) surgery after diagnosis.

DISCUSSION

General prevalence. Females and males diagnosed with tumor lesions accounted for 63.8% and 36.8%, respectively, which agrees with a previous study (32) reporting 58% lesions for females and 39% for males. We found that alterations of the mammary gland are the most frequent cause of consultation (1,5,12,13,33,34,35).

Prevalence by age, breed, and body condition. The prevalence of CMT was higher in purebreds, consistent with the results of other studies (9,10,23,24,35). Medium-sized and small breeds are more frequently diagnosed with CMT than large breeds (10,35). French Poodle is the most common among the purebreds, a finding that agrees with previous studies conducted in the same city (36) and other cities of Colombia (12,13). Cocker Spaniel and Beagle also show high prevalence (10). For large breeds, Labrador Retriever, Golden Retriever, and German

Shepherd were the most reported (9,11,12,32). The presence of CMT and its frequency in certain breeds greatly depends on the popularity of the breeds, degree of ownership, and trends for certain breeds in a given area. For this reason, no general tendency was observed. However, in small breeds, an earlier diagnosis of tumor alterations is more frequent since these breeds are more easily managed by their owners.

The mean age was 9.3 years with a higher prevalence between 10 and 12 years old. Our findings agree with previous studies (11,36,37) and with age ranges reported by different authors, 9-11 years old (4,20), 6-10 years (9,35), 7-11.9 years (33), 10-11.9 years (34), 8-11 years (32), and 8-10 years (13). In particular, six years old is known as the "age of cancer" (1,15,19). Furthermore, the incidence of the disease increases with age, with maximum peaks between 9 and 10 years old (1,35) and decreases around 12 years old. Another peak is observed at approximately 13 years old and there are also exceptional cases before 5 years old. The age of 10 in canine females is considered equivalent to 58 in women, and these are the mean ages of cancer presentation in both groups (38).

Clinical aspects and prevalence. The inguinal pairs (pair 5) were the most diagnosed with CMT (16.9%). Several findings indicate that pairs 4-5 were the most susceptible to CMT presentation (7,10,37). CMT is less frequent in the cranial thoracic glands. The affected area can involve a single gland (10) or the inguinal and cranial glands simultaneously (24). The patients showed greater compromise of the left side (7), but these findings differ from other studies (e.g., 9). Conventionally, the size of the mammary glands tends to decrease from the cranial to the caudal; in particular, the thoracic glands are the smallest, the abdominal glands are intermediate, and the inguinal are the largest. However, tumor presentation depends on other factors as well, including a change in the lymphatic drainage pattern through lymphangiogenesis during CMT development (39).

The diameters T1 and T3 were the most frequent, although we did not find patterns for diameter or clinical stage that indicated a dominance of any (13,35) since both displayed relatively similar percentages of presentation for the patterns of metastasis, LN or distant (7).

Among benign tumors, we mainly found tumors of epithelial origin such as benign mixed tumors, complex adenomas, fibroadenomas, and papillary adenomas (11). The most frequently diagnosed malignant tumors of epithelial origin were carcinoma in mixed tumor, tubular carcinoma and, papillary carcinoma. Other authors have also reported these tumors as the most prevalent (12). Inflammatory lesions can be confused with tumor alterations (40). Histopathologically, mammary inflammations are characterized by mononuclear and mixed infiltrates. Small and hard masses are usually benign, while large masses that tend to ulcerate are generally malignant (9). The survival rate is favored by surgical intervention (7,17), yet it can also be affected by other factors.

This study provides an update of the epidemiological variables of CMT and discusses the risk factors that affect the development of the disease. The findings are highly relevant considering the importance of canines as sentinels for CMT in humans. We identify the need and opportunity of continuing to develop epidemiological studies that correlate environmental factors with patterns of CMT occurrence. Furthermore, the registration, management, and definitive histopathological diagnosis of mammary neoplasia must be improved. This can be achieved by optimizing and integrating medical record storage systems to enable data management and analysis at the population level. Unless this is accomplished, it will be difficult to determine the real impact of CMT on public health.

Conflict of Interests

The authors declare that there are no conflicts.

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CAPÍTULO 3

ELISA AND FTIR: HER2 GENE EXPRESSION IN BLOOD SERUM OF CANINES WITH MAMMARY TUMOR

ELISA AND FTIR: HER2 GENE EXPRESSION IN BLOOD SERUM OF CANINES WITH MAMMARY TUMOR

Alejandro Clavijo-Maldonado [1,4]*; Enio Ferreira [2]; Jorge E. Pérez-Cardenas [3]; Carlos Vargas-Hernández [4]; Fredy A. Rivera-Páez [1]

[1] Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Calle 65 No. 26-10 - A.A. 275, Manizales, Colombia.

[2] Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 486 31270-901, Belo Horizonte, Minas Gerais, Brasil.

[3] Departamento de Ciencias Básicas, Facultad de Ciencias para la Salud, Universidad de Caldas, Calle 65 No. 26-10 - A.A. 275, Manizales, Colombia.

[4] Grupo de Propiedades Ópticas de Materiales, Departamento de Física y Química, Universidad Nacional de Colombia Sede Manizales, Manizales, Colombia.

*Autor de correspondencia: alejandrocmvz@misena.edu.co

Abstract:

Cancer is characterized by abnormal tissue growth that lacks regulation mechanisms and shows invasive capacity. The origin of this pathology is attributed to multiple factors and its development involves the accumulation of multiple genotypic abnormalities. These characteristics lead to diagnostic challenges, especially in the early stages of cancer. Cancer research based on blood biomarkers allows obtaining information on pathologic progression and therapeutic monitoring. This study applied ELISA and FTIR assay techniques identify the HER2 gene expression in blood serum from female dogs and to characterize the biochemical composition. ELISA tests assess the stage of primary tumor development and evolution and FTIR allows completely characterizing the biomolecules associated with the tumoral process. We analyzed blood serum samples from 20 female dogs (14 diagnosed with mammary tumor and six healthy dogs). We detected the concentrations of the HER2/*neu* protein using two ELISA kits for

canine and human detection, respectively. Infrared spectroscopy (IR) was conducted in absorbance mode at a frequency range of 400-4000 cm^{-1} and a resolution of 4 cm^{-1}, 50 scans. We determined the ELISA cut-off for HER2 protein concentration in blood serum using the receiver operating characteristic (ROC) curve and by estimating the area under the curve (AUC) at a 95% confidence interval (CI=95%). The ROC curves in the canine and human ELISA tests were 0.75 and 0.45, respectively. The representative IR spectra for HER2 gene expression corresponded to lipids (1161 cm^{-1}, 1452 cm^{-1}, 2851 cm^{-1}). This study contributes to the knowledge of HER2, through the identification of the biochemical features associated with the changes in the HER2/*neu*+ and HER2/*neu*- states.

Keywords: ErbB2, infrared spectroscopy, immunoassay, oncoprotein, blood.

1. Introduction:

Canine mammary tumor (CMT) is a common pathology in females (Moe, 2001; Zatloukal et al. 2005; Vacellari et al. 2009; Gal et al. 2016) and is characterized by high mortality levels (Silva et al. 2014), wherein one out of four dogs over two years old may die of this cancer (Hemanth et al. 2015). Growth factors (GFs) are polypeptides that stimulate cellular proliferation through high-affinity binding to membrane receptors (Goustin et al. 1986). This signaling process leads to autocrine growth phenomena, which are typical of neoplastic transformation (Surmacz, 2003; Witsch et al. 2010). Human epidermal growth factor receptors (EGFR/HER) are part of a family of receptor tyrosine kinases (RTK) (Biscardi et al. 2000). EGFR comprise a family of four members, including HER1 (ErbB1), HER2/*neu* (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (Ceballos & Hernández, 2008). The HER2 proto-oncogene is located in chromosome17q21 in humans (Fukushige et al. 1986) and 1q13.1 in canines (Murua-Escobar et al. 2001). This gene encodes for the 185-kDa transmembrane protein HER2/*neu* (Akiyama et al. 1986) that contains an intracellular tyrosine kinase domain (ICD), a transmembrane domain, and an extracellular domain (ECD) with a mass of 105-kDa, which is measurable in blood serum (Ha et al. 2014; Di Gioia et al. 2015). Active HER2 receptors stimulate cellular proliferation and tumor progression (Gutiérrez & Shiff 2011; Ressel et al. 2013), but not necessarily tumor malignization

(Rungsipipat et al. 1999; Martin de las Mulas et al. 2003; Carney et al. 2004; Ressel et al. 2013; Ferreira et al. 2014; Silva et al. 2014; Burrai et al. 2015; Campos et al. 2015).

HER2 is highly important in the prognosis of human breast cancer (HBC) development (Savino et al. 2009) since it is associated with resistance to chemotherapy and hormonal treatments, leading to reduced survival of patients with overexpression of this gene (Surmacz, 2003; Ceballos & Hernández, 2008). In canines, SNP-based studies have shown that mutations in HER2 are silent and could represent a natural variation in this gene (Hsu et al. 2009). However, silent SNPs can affect the kinetics of protein synthesis and generate diverse configurations of the final proteins (Kimchi-Sarfaty et al. 2007; Komar, 2007), ultimately challenging diagnosis and follow-up. The use of serum and blood-based biomarkers in cancer studies allows achieving a broad perspective of the biochemical characteristics and dynamics of cancer; furthermore, these markers are especially relevant when biopsies are difficult to perform (Jesneck et al. 2009). The enzyme-linked immunosorbent assay (ELISA) is used to evaluate the stage of primary tumor development by enabling to track changes in HER2 during the evolution of the mammary tumor (Carney et al. 2004). In humans, a serum HER2 (sHER2) concentration of 15 ng/mL is the most appropriate test cut-off value to measure the levels of this protein in blood (Tse et al. 2005; Fehm et al. 2007; Zhang et al. 2018), yet other authors have determined a cut-off of 22 ng/mL (Savino et al. 2009). Despite this, due to the wide variations in sHER2 levels, its clinical value is prognostic, particularly related to tumor progression and therapeutic response. (Carney et al. 2004; Carney et al. 2013). In canines, the diagnostic cut-off has not been accurately determined to date (Campos et al. 2015).

Fourier transformed infrared spectroscopy (FTIR) is a simple and rapid reagent-free method that is non-destructive and requires a small amount of sample (Zelig et al. 2015). Most cancer lesions are identified at the spectral region between 800 cm^{-1} and 1800 cm^{-1} (fingerprint); this range comprises the majority of functional groups, such as carbonyl, carbon-nitrogen, amino, methyl, methylene, carboxy, among others (Lima et al. 2015; Ferreira et al. 2020). However, the spectral region from 2800 cm^{-1} to 3000 cm^{-1}, called the first lipid region (Gavgiotaki et al. 2016), is also of interest due to its relevance in the study of HER2.

Considering the discriminant capacity of the ELISA technique to detect specific proteins and the possibility of identifying these molecules in blood serum, this study focused on the gene expression of HER2 in canine serum by identifying the expression features of the protein through

ELISA tests, as well as establishing the biochemical characteristics based on functional groups analysis through FTIR.

2. Materials and methods:

2.1. Sample collection and conservation

Between May of 2018 and March of 2019, we obtained samples from female dogs diagnosed with CMT (n=14) and healthy females (n=6). At the time of sample collection, not all patients had a definitive diagnosis of the tumor type . We collected 4 mL of blood through aseptic venipuncture using an evacuated tube without anticoagulant (BD Vacutainer, Becton Drive), with a clot retraction time of three hours at room temperature. The samples were then centrifuged at 3000 rpm for 10 minutes (Tuck et al. 2009). The resulting serum was stored at -80°C and multiple cycles of freezing and thawing were avoided. The samples with some type of deterioration were discarded.

2.2. ELISA – measurement of HER2 protein levels in blood serum

We used the Canine Epidermal Growth Factor Receptor 2 (Her2Ab) ELISA kit ref. MBS2606515 (MyBioSource, Southern California, San Diego - USA) and the Human HER2 Platinum ELISA kit ref. BMS207-2 (eBioscience, Vienna, Austria) to detect and quantify HER2 protein levels in blood serum. The assays were done according to the manufacturer's instructions. All assays were performed in duplicate, including the control with known concentrations (soluble fragment of the HER2 protein ($p185^{HER2}$) and a canine standard (HER2)). We used a Rayto RT-2600c (Guangming, Shenzhen, China) microplate reader for the measurements.

The prognostic criteria for classifying the patients with overexpression, according to the cut-off values were: 1. Patients with CMT and measurement of sHER2 equal to or above the cut-off: true positive (TP = CMT - HER2/*neu*+); 2. Control patients with a measurement of sHER2 below the cut-off: true negative (TN = Healthy - HER2/*neu*-); 3. Patients with CMT and measurement of sHER2 below the cut-off: false negative (FN = CMT - HER2/*neu*); 4. Control patients with a measurement of sHER2 equal to or above the cut-off: false positive (FP = Healthy - HER2/*neu*+).

2.3. Acquisition of FTIR spectra

We used 5 µL of blood serum at room temperature for FTIR. The IR spectra were acquired using an Alpha ATR Platinum (Bruker Corporation, Germany) spectrometer. The optimal measurement conditions were a room temperature of 20°C and 40% relative humidity. For the readings, each sample was placed on a diamond crystal tip. The reading parameters were absorbance mode, frequency range 400-4000 cm^{-1}, and resolution of 4 cm^{-1} with 50 scans per sample. We used readings from water as blank runs that were subsequently subtracted from the spectra of the samples (Elmi et al.2017; Santos et al. 2019; Macotpet et al. 2020).

2.4. Spectral data processing

Processing and analysis of the original spectra were done using OriginPro 9.0.0 (OriginLab Corporation, Northampton, MA 01060 USA). Each spectrum was normalized and baseline correction was performed according to the Savitzky–Golay method with a 2^{nd} order polynomial and 20 data points (Lima et al. 2015; Zelig et al.2015; Macotpet et al. 2020). The prognostic criteria of cut-off values from the ELISA tests, were used to identify the characteristics of the spectra for HER2/*neu* expression. The frequency ranges selected for this analysis included the fingerprint region, 800 cm^{-1} to 1800 cm^{-1} (Ghimire et al. 2020; Macotpet et al. 2020), which was divided into four subregions (800 cm^{-1} – 1100 cm^{-1}, 1100 cm^{-1} – 1380 cm^{-1}, 1380 cm^{-1} – 1700cm^{-1}, 1700 cm^{-1} – 1800 cm^{-1}), and a spectral region from 2800 cm^{-1} to 3000 cm^{-1} since it is relevant for the study of HER2/*neu* (Gavgiotaki et al. 2016;Ferreira et al. 2020).

The segments of the selected spectra were analyzed based on second-order derivatives to allow detecting minor peaks in bands from the main spectral regions and differentiate between patterns from ill and healthy individuals (Zeling et al. 2015; Ghimire et al. 2020). For this, the spectra were softened using the Savitzky–Golay method with a 2^{nd} order polynomial and 20 data points. The area under the curve (AUC) for each spectrum (original and second-order derivative) was calculated mathematically into a polygon area with an absolute measurement type. The functional groups and vibrational modes were assigned according to previous findings by several authors. The AUC ratio was expressed as the relative area under the curve (RAUC).

2.5. Statistical analysis

We analyzed the following accuracy parameters to determine the optimal ELISA cut-off: from the ROC curve, Youden's index (J) (i.e., the maximum vertical distance between the ROC curve and the diagonal (Schisterman et al. 2005)), sensitivity (Sn) and specificity (Sp), and minimum distance to the top-left corner (i.e., the minimum distance between the ROC curve and the left upper corner) (Hajian-Tilaki, 2013) was identified. The area under the curve (AUC) was determined to estimate the discriminant capacity of each assay. The prognostic criteria for classification of patients according to TP, TN, FP, and FN allowed establishing the predictive positive value (PPV) and predictive negative value (PNV).

The absorbance values obtained from the canine and human ELISA tests and the IR spectra (original and second derivative) were checked for normality using Shapiro-Wilks test. Based on the results from the normality test, we applied parametric tests when normal distribution was assumed and non-parametric tests for non-normal distributions. We applied a confidence interval of 0.95 and a p-value <0.05 was considered to be statistically significant. The statistical analyses were done using R v.3.2.2, NCSS 11 (NCSS, LLC. Kaysville, Utah, USA), and OriginPro 9.0.0.

2.6. Ethical aspects

This study was conducted with the approval of the Ethics Committee on Animal Experimentation, Faculty of Agropecuary Sciences, Universidad de Caldas (CEEA Código-15061601). All dog owners authorized the collection of samples from canine patients through informed consent.

3. Results:

3.1. Patients

The samples analyzed corresponded to seven breeds: Maltese, Miniature Schnauzer, Cocker Spaniel, French Poodle, English Bulldog, Pitbull, Samoyed, and mixed-breed. The average age was 9.3 years old.

ELISA

Based on the ROC curves, the optimal cut-off and AUC for the canine and human ELISA tests were ≥0.31 ng/mL and 0.75, ≥9.26 ng/mL and 0.45, respectively (Table 1, Figure 1). The PPV and PNV of canine and human ELISA test was 83%, 50% and 78%, 36%, and the accuracies for each test were 70% and 55%, respectively. Out of 20 female dog samples, the canine ELISA tests discriminated that 14 were HER2/*neu*+ (TP=71%, FP=29%) and six HER2/*neu*- (TN=67%, FN=33%). Moreover, the human ELISA tests yielded TP=50%, FP=50%, TN=67%, and FN=33%. The Shapiro-Wilks normality test showed statistically significant differences ($p<0.05$), except for ELISA HER2-negative patients ($p= 0.13$).

Table 1. Accuracy parameters and optimal cut-off for the canine-ELISA and human-ELISA tests.

Parameter	Test	
	Canine ELISA	Human ELISA
Cut-off Value	≥ 0.31 ng/ml	≥ 9.26 ng/ml
Sensibility (%)	71	50
Specificity (%)	67	67
PPV (%)	83	78
PNV (%)	50	36
Accurancy (%)[a]	70	55
95% Cl-Sensibility		
Lower	0.419	0.2304
Upper	0.9161	0.7696
95% Cl-Specificity		
Lower	0.2228	0.2228
Upper	0.9567	0.9567
J (95% CI)[b]	0.381	0.1667
Sensibility + Specificity	1.381	1.166
Distance to Corner	0.439	0.6009

[a]Accuracy = Proportion of correctly classified patients
[b]J = Youden Index. Sensibility + Specificity-1.

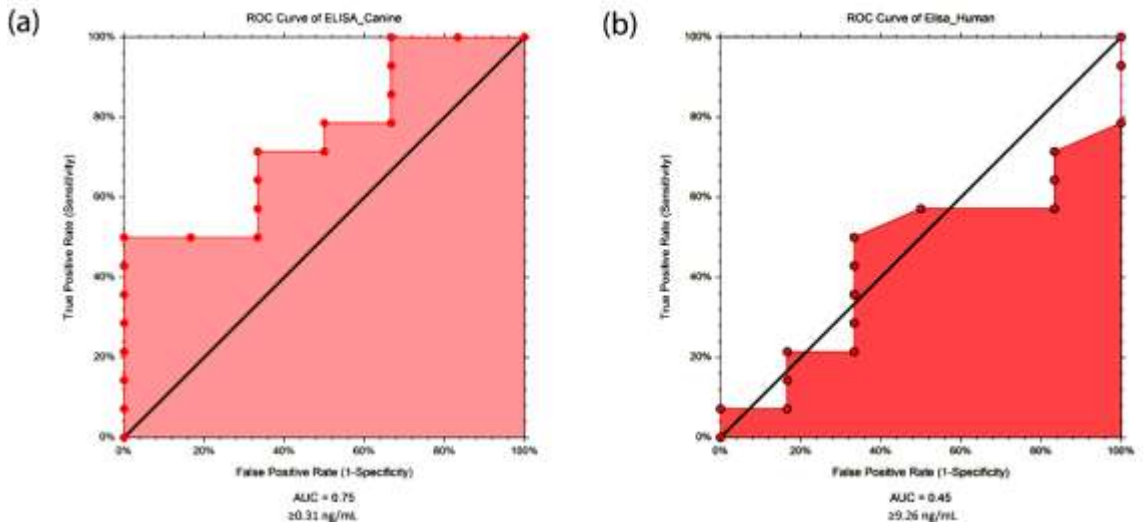

Figure 1. ROC curves for canine and human ELISA tests. (A) ROC curve for the canine protein ELISA (Her2Ab), AUC = 0.75 (CI 95%). Concentration of the protein in blood serum (0.27 ng/ml). (B) ROC curve for the human protein ELISA, AUC = 0.45 (CI 95%). Concentration of the protein in blood serum (8.98 ng/ml).

3.2 FTIR spectra in blood serum in HER2/*neu* positive-negative female dogs

The study of HER2-positive and negative states was based mainly on the similar results between the canine and human ELISA tests, which showed that TP and FN individuals display malignant and benign lesions, such as mammary carcinoma and benign adenoepithelioma, whereas TN and FP females are clinically healthy. The original infrared spectra from blood serum (Figure 2) show six main spectral peaks in ranges 800 cm^{-1} to 1800 cm^{-1} and 2800 cm^{-1}, corresponding to proteins, esters, nucleic acids, phospholipids, and lipids.

The peaks with the highest absorbance are associated with clinically healthy HER2/*neu*-positive females. Table 2 summarizes the vibrational modes assigned and the corresponding blood serum composition according to several authors.

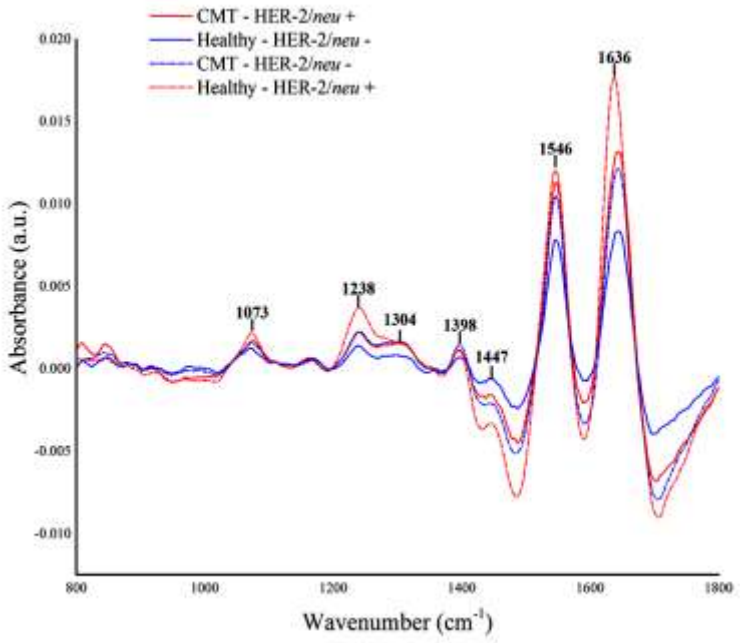

Figure 2. FTIR original spectra for females with CMT and healthy female dogs. Absorbance bands of the main functional groups at frequency ranges from 800 cm^{-1} to 1800 cm^{-1} for HER2/*neu* positive females (red lines) and HER2/*neu* negative females (blue lines).

Table 2. Main vibrational modes assigned to the original FTIR spectra in canine blood serum (described in Figure 2) according to various references.

Spectra (cm^{-1})	Organic compound/Vibrational mode assignment	References
1073	Phospholipids: $v_s(PO_2)$	Oleszko et al. 2015
1238	Protein: Amide III	Ferreira et al. 2020
1304	Lipids	Bi et al. 2014; Depciuch et al. 2016
1398	Phospolipids/fatty acids, amino acids: $v_s(COO^-)$	Kar et al. 2019
1447	Protein (methyl groups), lipids: CH_3 asymmetric bending [δas (CH_3)]	Kar et al. 2019; Ferreira et al. 2020
1546	Protein: Amide II [*v* (N–H) *v* (C–N)]	Ferreira et al. 2020
1636	Protein: Amide I [*v* (C=O), *v*(C–N), δ (N–H)]	Ferreira et al. 2020; Ghimire et al. 2020

v = stretching vibrations, δ = bending vibrations, s = symmetric vibrations, as = asymmetric vibrations.

The second-order derivatives of the infrared spectra allowed identifying 13 additional representative peaks assigned to four subregions. Specifically, subregion 1 (800 cm^{-1} – 1100 cm^{-1}) included phospholipids, tyrosine-protein, esters; subregion 2 (1100 cm^{-1} – 1380 cm^{-1}) included amide III, carbohydrates, lipids; subregion 3 (1380 cm^{-1} – 1700cm^{-1}) corresponded to phospholipids, fatty acids, lipids, amide I, amide II, and tyrosine protein; and subregion 4 (1700

cm^{-1} – 1800 cm^{-1}) showed lipids and esters. Additionally, region 2800 cm^{-1} to 3000 cm^{-1} was characterized by lipids, phospholipids, and cholesterol (Figure 3). The description of the vibrational modes and the corresponding functional groups is shown in Table 3.

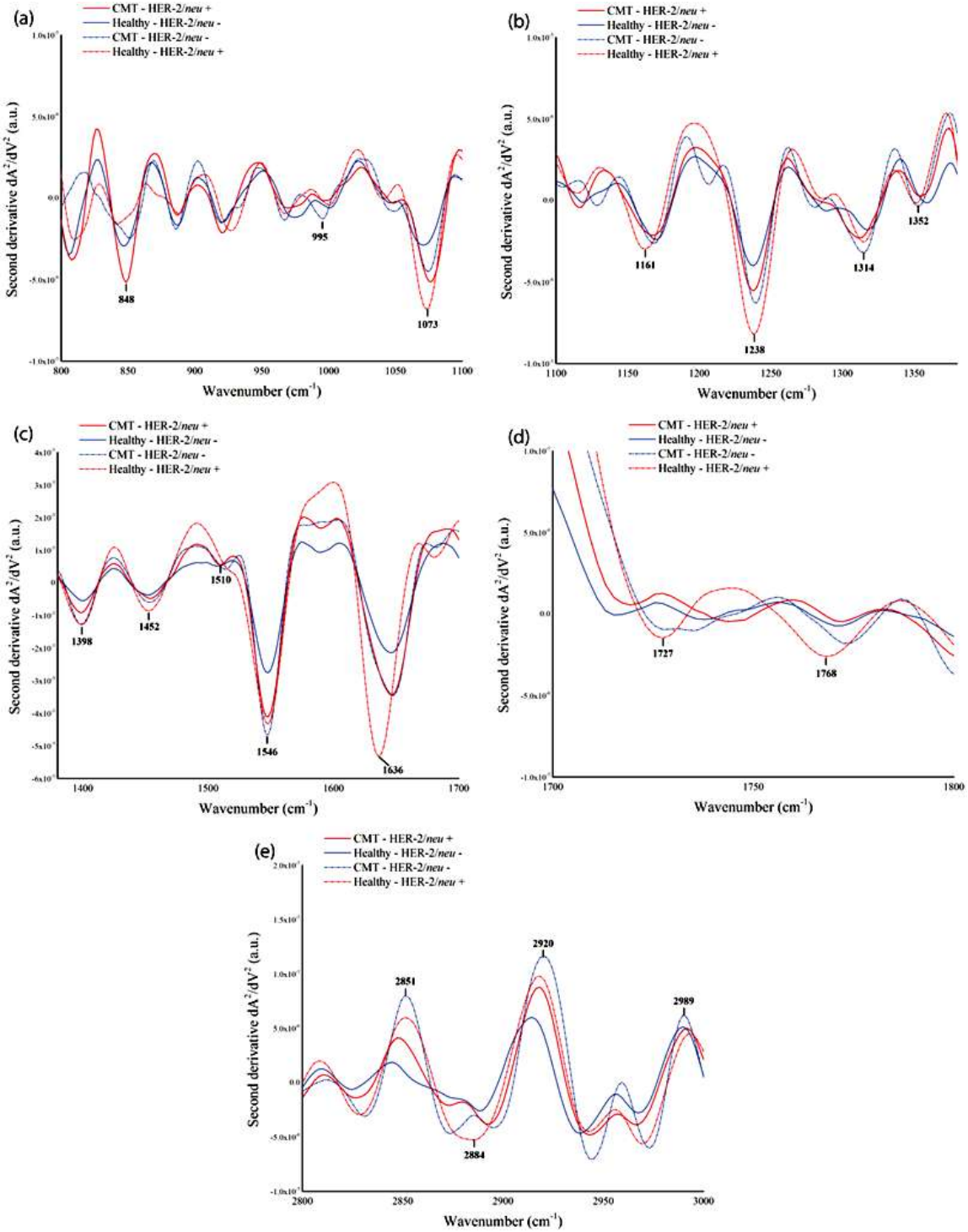

Figure 3. Detail of the second-derivative spectra and most representative frequencies. Spectra (a) 800 cm^{-1} – 1100 cm^{-1}, (b) 1100 cm^{-1} – 1380 cm^{-1}, (c) 1380 cm^{-1} - 1700 cm^{-1}, (d) 1700 cm^{-1} - 1800

cm^{-1}, (e) 2800 cm^{-1} – 3000 cm^{-1} for HER2/*neu* positive females (red lines) and HER2/*neu* negative females (blue lines).

Table 3. FTIR peaks of the average second-derivative spectra of canine HER2/*neu* according to various references.

2nd-derivative Spectra (cm^{-1})	Organic compound/Vibrational mode assignment	References
848	Tyrosine proteins	Bi et al. 2014; Zelig et al.2015; Depciuch et al. 2016
995	Ester bands: vs(C-O)	Elmi et al.2017; Ferreira et al. 2020
1161	Protein/Carbohydrate/lipid ester bonds: CO–O–C asymmetric stretching (vas (CO-O-C))	Ferreira et al. 2020; Oleszko et al. 2015
1314	Protein: Amide III	Ferreira et al. 2020
1352	Protein: Amide III	Elmi et al.2017
1452	Lipid and protein: Methylene bending	Bi et al. 2014; Ferreira et al. 2020
1510	Tyrosine proteins: ν(C-C)	Kar et al. 2019
1727	Lipids/ester bands:	Gavgiotaki et al. 2016
1768	Ester	Elmi et al.2017
2851	Lipids, long-chain fatty acids: vs(CH2)	Oleszko et al. 2015
2884	Lipid: CH3 asymmetric stretching (vas (CH3))	Elmi et al.2017; Ferreira et al. 2020
2920	Nucleic acid/Lipids, long-chain fatty acids: CH2 asymmetric stretching (vas (CH2))	Kar et al. 2019; Ferreira et al. 2020
2989	Phospholipids/cholesterol:	Gavgiotaki et al. 2016

ν = stretching vibrations, δ = bending vibrations, s = symmetric vibrations, as = asymmetric vibrations.

We established four groups according to the type of spectra from 20 peaks (original and second derivatives): group 1, proteins; group 2, lipids; group 3, esters; and group 4, mixed. We did not find statistically significant differences within each group and the prognostic categories (Table 4).

Table 4. Statistical analysis of FTIR peaks of the average second-derivative spectra for diagnostic categories and compositional groups.

Group	**Original-2nd derivative peak (cm^{-1})**	**TP (Mean±SD)**	**TN (Mean±SD)**	**FP (Mean±SD)**	**FN (Mean±SD)**	**ANOVA (*p-value*)[1]**	**t-test (*p-value*)[2]**	**Tukey-test (*p-value*)[3]**	**Levene´s-test (*p-value*)[4]**
Proteins	848, 1314, 1238, 1352, 1510, 1546, 1636	2.15E-6 ± 3.78E-6	1.34E-6 ± 3.59E-6	3.73E-6 ± 5.96E-6	1.59E-6 ± 5.99E-6	0.21	> 0.05	> 0.05	1.5
Lipids	2851, 2884, 2989	-1.06E-5 ± 1.66E-5	-6.70E-6 ± 1.12E-5	-1.13E-5 ± 1.85E-5	-1.43E-5 ± 2.37E-5	0.14	> 0.05	> 0.05	0.9
Ester	995, 1073, 1304, 1768	-1.68E-6 ± 2.18E-6	-1.08E-6 ± 1.17E-6	-2.12E-6 ± 1.56E-6	-2.56E-6 ± 3.03E-6	0.35	> 0.05	> 0.05	0.8
Mixed	1161, 1398, 1447, 1452, 1727, 2920	-1.56E-6 ± 5.91E-6	-1.41E-6 ± 3.45E-6	-2.38E-6 ± 8.02E-6	-3.96E-6 ± 7.68E-6	0.18	> 0.05	> 0.05	0.6

Abreviations: TP: CMT - HER-2/*neu*+, TN: Healthy - HER-2/*neu*-, FN: CMT - HER-2/*neu*-, FP: Healthy - HER-2/*neu*+
Note: [1] Represents $p>0.05$ to comparisons of all prognostic classifications; [2] Represents $p>0.05$ to comparisons TP vs. TN, TP vs. FP, TN vs. FN
[3] Represents $p>0.05$ indicates that the difference of the means in not significant; [4] Represents $p>0.05$ homogeneity of variance are not significantly different

The RAUC showed differences between states, indicated by a higher abundance of peaks corresponding to lipids for HER2/*neu*+ (1161 cm^{-1}, 1398 cm^{-1},1452 cm^{-1}, 2851 cm^{-1}, 2920 cm^{-1}), except at 1447 cm^{-1} and 2884 cm^{-1} and 1304 cm^{-1} peak and amide I (1636 cm^{-1}). The RAUC for tyrosin-type proteins (848 cm^{-1}, 1510 cm^{-1}), amide III (1314 cm^{-1}, 1352 cm^{-1}), and amide II (1546 cm^{-1}) were greater for HER2/*neu*-, as well as esters (995 cm^{-1}, 1768 cm^{-1}) and phospholipids at 2989 cm^{-1}. These findings indicate that amide I and lipid-related functional groups are a product of HER2 gene overexpression. Table 5 shows the degree of comparative accuracy between the ELISA tests and FTIR.

Table 5. Prognostic accuracy of ELISA – FTIR for CMT patients.

Diagnostic criteria	ESLISA test		FTIR (%)
	Canine ELISA (%)	Human ELISA (%)	
TP	50	35	65
FP	10	10	0
FN	20	35	5
TN	20	20	30
CCP[a]	60	45	65
ICP[b]	40	55	35
Sensibility	71	50	93
Specificity	67	67	100

TP: True positive = CMT - HER-2/*neu* +
FN: False positive = CMT - HER-2/*neu* -
FP: False negative = Healthy - HER-2/*neu* +
TN: True negative = Healthy - HER-2/*neu* -
[a] Correctly Classified Proportion
[b] Incorrectly Classified Proportion

4. Discussion:

This study contributes to the knowledge of HER2 regarding gene expression and detection in blood serum, its clinical relevance in CMT research, and its application as a model for human breast cancer (HBC). We demonstrated the likelihood of using canine and human antigens to determine and compare the sHER2 concentrations. The canine ELISA tests yielded TP = 71%, TN = 67%, FP = 33%, and FN = 29%, whereas the human ELISA test showed 50%, 67%, 33%, and 50%, respectively. Although there is high homology between human and canine HER2 antigens (Singer et al. 2012), our findings demonstrate that the use of the human HER2 protein is not adequate to evaluate the concentration of this protein in canine serum, in agreement with reports by Campos et al. (2015). This is likely due to the low capacity of canine antibodies to

recognize human HER2. Findings indicate that the antigenic determinants from the two molecules are not the same perhaps due to differences in the tertiary structure of the proteins (Kimchi-Sarfaty et al. 2007; Komar, 2007; Hsu et al. 2009) or cross-reaction with other members of the HER family (Burrai et al. 2015). Both tests coincided in 29% (4/14) of TP females, TN 33% (2/6), FN 50% (3/6), FP 7% (1/14). Our results on the overexpression of the HER2/*neu* protein are similar to reports from previous studies (Dutra et al. 2004; Ressel et al. 2013; Shinoda et al. 2014; Campos et al. 2015).

A positive correlation has been determined between the presence of HER2 in blood serum and tumor tissue (Campos et al. 2015), tumor mitotic index, high histological grade and size (Muhammadnejad et al. 2012; Silva et al.2014), although no significant differences have been found between HER2 expression in benign and malignant tumors (Kim et al. 2011;Ressel et al. 2013), indicating that HER2 may participate in tumor formation and rapid progression of CMT (Dutra et al, 2004; Ferreira et al. 2009; Bertagnolli et al. 2011), but not necessarily in malignant transformation, or at least, it is not a good marker of malignancy (Kaszak et al. 2018). Although, the clinical association of HER2 is controversial (Hsu et al. 2009; Ressel et al. 2013; Burrai et al. 2015; Campos et al. 2015). Unfortunately, in this study it was not possible to compare the ELISA and FTIR findings with histological parameters due to limited availability of biopsies.

Based on infrared spectroscopy, we analyzed diverse biochemical patterns, such as proteins, lipids, esters, nucleic acids, and carbohydrates. Although we did not obtain significant differences, distinct features between HER2/*neu*-positive and negative states were observed for certain functional groups, such as the relative intensity at peak 1636 cm^{-1}. Furthermore, this study analyzed an additional region between 2800 cm^{-1} and 3000 cm^{-1} that had not been studied in CMT. Previous research on HER2 using infrared spectroscopy, among other biochemical analysis techniques in cells, tissues, and fluids, report changes in the compositional profile, especially regarding lipids (CH_2, CH_3), which are greater in the HER2/*neu*-positive state (Hartsuiker et al. 2010; Bi et al. 2014; Gavgiotaki et al. 2016). These reports agree with the findings reported here, except for peaks 1304 cm^{-1}, 1447 cm^{-1}, and 2884 cm^{-1}. Moreover, high contents of lipids in cytoplasmic organeles are reported in HER2+ HBC cell cultures (Hartsuiker et al. 2010; Gavgiotaki et al. 2016), which are involved in the adhesion and migration of epithelial tumor cells (Murai 2012). Similarly, several studies have detected an increase in phospholipids, associated with fatty acids and cholesterol synthesis (Menendez & Lupu 2007; Bi

et al. 2014; Elmi et al. 2017; Kar et al. 2019). This pattern was only observed at peak 1398 cm^{-1} (phospholipid, fatty acid, and amino acid complex). This phenomenon is caused by the regulation of the fatty acid synthase associated with a low regulation of tyrosine kinase receptors (Jin et al. 2010).

On the other hand, we report an increase in amide I, especially in HER2/*neu*+. However, amide I not necessarily is associated with HER2 overexpression. Several authors report an increase in this protein in HBC (Elmi et al. 2017; Chrabaszcz et al. 2018), but a consistent variation is not observed in HER2+ cell cultures (Bi et al. 2014). Amide I and amide II are reported in greater amounts in hyperplastic tissues (Tian et al. 2015); however, a reduction of amide II and an increase of amide I suggest a possible malignization process (Simonova & Karamancheva 2014). A reduction of amide III has been observed in HER2+ cell cultures (Bi et al. 2014), which is consistent with our findings.

FTIR enables the non-invasive recognition of tissue alterations through markers of cellular activity. In this study, we detected a Sn 93% and Sp 100%. This agrees with recent studies in dogs that identified the most important spectra in cancer using blood serum and reported a Sn and Sp of 76.7% and 87.5%, respectively (Macotpet et al. 2020) and Sn 100% and Sp 100% in conformational proteins in HBC (Ghimire et al. 2020).

On the basis of analogies between both species (Rowell et al. 2011; Pinho et al. 2012), and the homology between human and canine HER2 antigens, the immunotherapy seems to be promising in canine patients with HER-2 expression (Singer et al. 2012; Kaszak et al. 2018). However, additional markers associated with the expression of this gene must be considered in order to elucidate the prognostic value of HER2 overexpression in female dogs.

5. Conclusion:

Our study allowed recognizing important features of HER2 through discriminant ELISA tests and several biochemical characteristics based on spectral patterns in a biological fluid, such as blood serum. Future studies should address the amplification of the IR spectra described here using nanoparticles in blood serum from ELISA HER2/*neu*-positive or negative patients. Finally, the results of the FTIR identified two possible control peaks of HER2 overexpression, corresponding to the regions surrounding peak 1636 cm^{-1} and a band between 2800-3000 cm^{-1}, which were associated with amide I and the corresponding lipids (CH_2, CH_3), respectively.

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CONSIDERACIONES FINALES

Los resultados del presente estudio soportan que factores como razas puras, particularmente pequeñas y medianas, edad media comprendida entre los 10 a 12 años, sobrepeso y dietas caseras, poseen alto impacto en la incidencia y progresión patológica de neoplasias mamarias. El patrón clínico de presentación de TMC se caracterizó por una mayor afectación en los pares mamarios inguinales, sin un predominio de un tamaño tumoral especifico.

En humanos, niveles elevados de la proteína HER-2/*neu* (dominio extracelular) representan un alto valor diagnóstico, pronóstico y terapéutico, entre tanto que la relación entre la dinámica de la expresión de HER-2 y la incidencia de TMC y sus patrones de presentación no son claros. En el presente estudio, la sobreexpresión del gen HER-2 no presentó relación estrecha con la condición clínica de base de diagnóstico TMC y clínicamente sana. Sin embargo, la interacción entre la capacidad discriminante de ELISA junto con la sensibilidad de la técnica FTIR para describir las características bioquímicas de las muestras de suero sanguíneo, permitió describir rasgos claves entre los estados HER/*neu*+ y HER-2/*neu*. Los picos IR correspondientes a amida I y lípidos, son indicativos de como picos control para la sobreexpresión del HER-2 y por lo tanto como un método para el estudio del dominio extracelular de la proteína de este gen. No obstante, y como lo han referenciado otros autores, a pesar de que en caninos el desarrollo tumoral a nivel de glándula mamaria es agresivo, HER-2 no necesariamente actúa en el proceso de malignización, pero si en el de proliferación celular inicial, lo cual necesariamente debe aumentar su importancia como blanco de estudio e importancia diagnóstica. Lo anterior puede ser explicado en virtud de la estructura de la proteína HER-2/*neu* en caninos difiere de la humana debido al origen de las mutaciones detectadas mediante estudios en SNPs.

El presente estudio, contribuye con la tendencia, importancia y el uso de biomarcadores para el estudio de alteraciones neoplásicas a través de suero sanguíneo. Lo cual representa mínima invasión, manipulación y trauma al paciente, lo que es relevante cuando el estado de salud está deteriorado y las condiciones para toma de otro tipo de tejido no es factible. Por lo cual, el uso de fluidos biológicos y la exploración de biomarcadores tumorales, representan una alternativa factible en el establecimiento de protocolos eficientes de diagnóstico a la hora de definir una conducta terapéutica.

Estudios posteriores deben abordar variables epidemiológicas que correlacionen los factores ambientales con patrones de ocurrencia de TMC, lo que es especialmente importante al considerar la relevancia de los caninos como indicadores de la salud humana. Dada la alta complejidad que subyace en el monitoreo del gen HER-2 en hembras caninas, es importante considerar el uso de nanopartículas para la amplificación de señales de espectros IR empleando suero sanguíneo u otro tipo de fluido biológico. Del mismo modo, teniendo en cuenta el rol desempeñado por el gen HER-2 en la proliferación del tejido mamario, es preciso correlacionar la dinámica de la expresión del mismo frente a otros genes y factores intrínsecos de los pacientes, además de los aspectos clínicos.

ANEXOS

Anexo 1. Clasificación de estado clínico. Tomado y modificado de Owen, 1980.

Número de historia clínica: _____ Propietario: _____ Fecha: _____

Edad paciente: _____ Sexo: _____ Raza: _____ Peso: _____

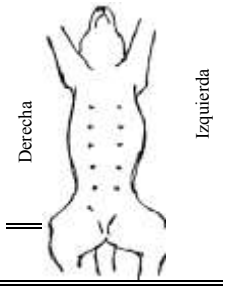
Número de tumores primarios:

	Cadena derecha	Cadena izquierda
Localización de tumores primarios en glándula mamaria	1 2 3 4 5	1 2 3 4 5
Tamaño tumoral	1 2 3 4 5	1 2 3 4 5
Diámetro (cm):	_____	_____

Único ☐
Múltiple ☐

Marcar lo que corresponda

Encerrar en circulo las glándulas mamarias involucradas



Requerimientos mínimos para la asignación de las categorías T, N y M

Categoría T: Valoración clínica y quirúrgica
Categoría N: Valoración clínica y quirúrgica
Categoría M: Valoración clínica, quirúrgica y radiografía de tórax

Categorización

T: Tumor Primario

T0	Sin tumor evidente	
T1	Tumor < 3 cm diámetro	
	No	
T1a	fijo	
T1b	Fijo en piel	
T1c	Fijo en músculo	
T2	Tumor 3-5 cm diámetro	
	No	
T2a	fijo	
T2b	Fijo en piel	
T2c	Fijo en músculo	
T3	Tumor > 5 cm diámetro	
	No	
T3a	fijo	
T3b	Fijo en piel	
T3c	Fijo en músculo	
T4	Cualquier tamaño tumoral	
	Carcinoma inflamatorio	

Los tumores múltiples son clasificados independientemente

N: Linfonodo Regional (LNR)*

		LRN evaluado: Inguinal	LRN evaluado: Axilar	Método de evaluación de LRN: Clínica	Método de evaluación de LRN: Histológica
N0	LRN no involucrado				
N1	LRN ipsilateral involucrado				
N1a	No fijo				
N1b	Fijo				
N2	LRN bilateral involucrado				
N2a	No fijo				
N2b	Fijo				

*LRN: nódulos inguinales y axilaress

M: Metástasis distante (MD)

		Método de evaluación: Clínica	Radiográfica	Histopatológica
M0	Sin evidencia de MD			
M1	MD con incluidos nódulos distantes			

Especificar sitios: _____

Agrupación de estados

	T	N	M
I	T1a, T1b, T1c	N0 (-), N1a (-), N2a (-)	M0
II	T0	N1 (+)	M0
	T1a, T1b, T1c	N1 (+)	
	T2a, T2b, T2c	N0 (+), N1a (+)	
III	Cualquier T3	Cualquier N	M0
	Cualquier T	Cualquier Nb	
IV	Cualquier T	Cualquier N	M1

Estado clínico:
Evaluación:

T	R	M
☐	☐	☐

Estado: _____

Observaciones: _____

Anexo 2. Comparación de cuatro sistemas de clasificación histológica de neoplasias mamarias en caninos.

Clasificación histológica - 1974[a]	Clasificación histológica - 1999[b]	Clasificación histológica - 2010[c]	Clasificación histológica - 2011[d]
I. Carcinoma	**1.** Tumores Malignos	**1.** Neoplásias Malignas Epiteliales	Tumores Malignos
A. Adenocarcinoma	**1.1.** Carcinoma (*in situ*) no infiltrante	Carcinoma - in situ	Carcinomas
1. Tubular	**1.2.** Carcinoma complejo	Carcinoma - simple	Carcinomas *in situ*
a. Simple	**1.3.** Carcinoma sumple	a. Tubular	Carcinoma ductal *in situ*
b. Complejo	1.3.1. Carcinoma tubulopapilar	b. Tubulopapilar	Carcinoma lobular *in situ*
2. Papilar	1.3.2. Carcinoma sólido	c. Quístico-papilar	Carcinoma mixto
a. Simple	1.3.3. Carcinoma anaplástico	d. Cribiforme	Carcinoma complejo o adenoepitelioma maligno
b. Complejo	**1.4.** Tipos especiales de carcinoma	Carcinoma-micropapilar invasivo	Carcinoma papilar
3. Papilar quístico	1.4.1. Carcinoma de células fusiformes	Carcinoma sólido	Carcinoma tubular
a. Simple	1.4.2. Carcinoma de células escamosas	Comedocarcinoma	Carcinoma sólido
b. Complejo	1.4.3. Carcinoma mucinoso	Carcinoma anaplastico	Tipos especiales de carcinomas
B. Carcinoma sólido	1.4.4. Carcinoma rico en lípidos	Carcinoma en adenoma complejo / tumor mixto	Carcinoma micropapilar
a. Simple	**1.5.** Sarcoma	Carcinoma complejo	Carcinoma lobular invasivo
b. Complejo	1.5.1. Fibrosarcoma	Carcinoma y mioepitelioma maligno	Carcinoma lobular pleomórfico
C. Carcinoma de células fusiformes	1.5.2. Osteosarcoma	Carcinoma mixto	Carcinoma secretorio
a. Simple	1.5.1. Otros sarcomas	Carcinoma ductal	Carcinoma mucinoso
b. Complejo	**1.6.** Carcinosarcoma		Carcinoma rico en lípidos
D. Carcinoma anaplástico	**1.7.** Carcinoma o sarcoma en tumor benigno	**2.** Neoplásias Malignas Epiteliales - tipos especiales	Carcinoma de células escamosas
E. Carcinoma de células escamosas		Carcinoma de células escamosas	Carcinoma de células fusiformes
F. Carcinoma mucinoso	**2.** Tumores Benignos	Carcinoma adenoescamoso	Carcinoma anaplástico
	2.1. Adenoma	Carcinoma mucinoso	Neoplásias mamarias con deferenciación sebacea
II. Sarcoma	2.1.1. Adenoma simple	Carcinoma rico en lípidos (secretorio)	Sarcomas
A. Osteosarcoma	2.1.2. Adenoma complejo	Carcinoma de células fusiformes	Fibrosarcoma
B. Fibrosarcoma	2.1.3. Adenoma basaloide	Mioepilioma maligno	Osteosarcoma
C. Osteocondrosarcoma	**2.2.** Fibroadenoma	Carcinoma de células escamosas - variante de células fusiformes	Carcinosarcoma
[fibro-lipo-Osteocondrosarcoma]	2.2.1. Fibroadenoma de baja celularidad	Carcinoma - variante de células fusiformes	Sarcoma mixto
D. Otros sarcomas	2.2.2. Fibroadenoma de alta celularidad	Carcinoma inflamatorio	Otros sarcomas
			Condrosarcoma puro
III. Carcinosarcoma (Tumor Mixto Mamario)	**3.** Tumores sin clasificación	**3.** Neoplásias Malignas Mesenquimales - sarcomas	Liposarcoma
		Osteosarcoma	Hemangiosarcoma
IV. Tumor benigno o aparentemente benigno	**4.** Hiperplásia y displásia mamaria	Condrosarcoma	
A. Adenoma	**4.1.** Hiperplásia ductal	Fibrosarcoma	Tumores Benignos
B. Papiloma	**4.2.** Hiperplásia lobular	Hemangiosarcoma	Adenoma
1. Papiloma ductal	4.2.1. Hiperplásia epitelial	Otros sarcomas	Adenoma complejo o adenomioepitelioma
2. Papilomatosis ductal	4.2.2. Adenosis		Adenoma basaloide
C. Fibroadenoma	**4.3.** Quistes	**4.** Carcinosarcoma - Tumor Mamario Mixto Mamario	Fibroadenoma
1. Pericanalicular	**4.4.** Ectasia ductal		Tumor mixto benigno
2. Intracanalicular	**4.4.** Fibrois focal (fibroesclerosis)	**5.** Neoplásias Benignas	Papiloma ductal
a. Tipo no celular	**4.5.** Ginecomastia	Aenoma simple	
b. Celular		Adenoma papilar intraductal (papiloma ductal)	Lesiones epiteliales no neoplásicas
3. Tumor mixto benigno		Adenoma ductal (adenoma basaloide)	Hiperplásia epitelial
4. Cambio fibroadenomatoso total		Fibroadenoma	Hiperplasia ductal
D. Tumor benigno de tejidos blandos		Mioepitelioma	Hiperplasia lobular
		Adenoma complejo (adenoepitelioma)	Adenosis
V. Tumores no clasificados		Tumor mixto benigno	Lesiones en células colimnares
			Alteración en células columnares
VI. Displasia benigna o aparentemente benigna		**6.** Hiperplásia/displásia	Hiperplasia de células columnares
A. Quiste		Ectasia ductal	Lesión atípica de células columnares
1. No papilar		Hiperplasia lobular	
2. Papilar		Regular	
B. Adenosis		Con actividad secretora (lactasional)	
C. Proliferación epitelial típica regular en ductos o lóbulos		Con fibrosis (fibrosis interlobular)	
D. Ectasia ductal		Con atípia	
E. Fibroesclerosis		Epiteliosis	
F. Ginecomastia		Papilomatosis	
G. Otras lesiones proliferativas no neoplásicas		Cambio fibroadenomatoso	
1. Hiperplasia lobular no inflamatoria		Ginecomastia	
2. Hiperplasia lobular inflamatoria			
		7. Neoplasias del pezón	
		Adenoma	
		Carcinoma	
		Carcinoma con infiltración epidermal (enfermedad tipo Paget)	
		8. Hiperplasia/displasia en el pezón	
		Melanosis en piel del pezón	

[a]Hampe & Misdorp 1974:
Clasificación basada en descripción morfológica y en poca proporción en histogénesis. Soportada en la clasificación de la OMS para tumores de seno en humanos (Scarff & Torloni, 1968).

[b]Misdorp et al. 1999
Sistema basado en la combinación de características nucleares de las células y aspectos histológicos

[c]Goldschmidt et al. 2011
Crea nuevas categorías por tipos histopatológicos y marcadores inmunihistoquímicos.

[d]Cassali et al. 2011
De acuerdo a marcadores tumorales se re categoriza la clasificación de Misdorp et al. (1999).

Anexo 3. Consentimiento informado a propietarios para toma de muestras de sangre de hembras caninas.

Alejandro Clavijo Maldonado, Médico Veterinario Zootecnista, Maestría en Ciencias Biológicas – Universidad de Caldas
Tel. +57 317 471 4976; email: clavijomal@gmail.com

Expresión del gen HER-2 y su diagnóstico en cáncer mamario de *Canis lupus familiaris* Linnaeus, 1758 (Canidae) en Manizales, Colombia. Versión 2, Abril, 2018.

INFORMACION PARA EL PACIENTE Y FORMATO DE CONSENTIMIENTO

El presente proyecto pretende evaluar la expresión del gen de crecimiento epidérmico tipo 2 (HER-2) su interacción con otros genes y su impacto diagnóstico en pacientes caninas hembras con tumores de glándula mamaria. Nuestra finalidad es estructurar un protocolo de alerta temprana en la glándula mamaria sin necesidad de la toma de biopsia. El presente Consentimiento Informado es para que usted y su mascota participen en una investigación para poder conocer sobre la dinámica de este gen mediante diversas técnicas de detección. Esta información nos ayudará a tomar mejores decisiones para el diagnóstico y el procedimiento clínico.

Su participación es absolutamente voluntaria.

Procedimientos del estudio

Si usted acepta participar, a su mascota se le tomará dos muestras de sangre de 4 ml cada una, lo que equivale a una jeringa llena de tamaño pequeño. Adicionalmente, su mascota será valorada clínicamente a través de la gestión de un formato de historia clínica en la cual se incluirá una valoración externa de las glándulas mamarias.

Beneficios

La información obtenida en este estudio podría ayudarnos en el futuro a mejorar el control y la prevención de esta enfermedad para otras pacientes.

Riesgos

La toma de la muestra de sangre se hará bajo condiciones de estricta limpieza para minimizar el riesgo de una infección posterior. En el procedimiento puede producirse un poco de dolor y quedar un pequeño morado que se resolverá sin tratamiento en las próximas dos semanas. La cantidad total de sangre necesitada es similar a la necesitada en las pruebas de laboratorio en sangre usuales y no representa un riesgo importante para la salud.

Responsabilidades del propietario

Usted debe permitir la realización de las pruebas, incluyendo la toma de muestra de sangre y los otros procedimientos tales como la valoración clínica. Usted ayudará mucho si comunica alguna variación o alteración que observe en su mascota.

Alternativas

No existe un método alternativo más seguro que permita obtener la información que estamos solicitando. La lectura de este consentimiento no le compromete automáticamente a participar de este estudio. Si usted escoge no participar en el estudio, su médico veterinario de cabecera proseguirá con el seguimiento clínico habitual se su mascota.

Confidencialidad

Sólo su médico veterinario de cabecera y sus colaboradores sabrán que usted está participando en el estudio. Los registros que se hagan se harán identificándolo sólo con un código y no con el nombre; sin embargo, representantes autorizados de las autoridades reguladores podrán revisar sus registros como parte de su actividad de supervisión del estudio. Si los resultados de este estudio son publicados, usted ni su mascota no serán identificados por el nombre.

Compensación

Usted no tendrá que incurrir en ningún gasto por participar en este estudio. Las jeringas así como el recipiente de colección y almacenamiento de la sangre, además de las pruebas son cubiertas completamente por el estudio. Si como consecuencia directa de los procedimientos de este estudio sufre enfermedades o daños físicos, se le proveerá el cuidado profesional médico veterinario.

Alejandro Clavijo Maldonado, Médico Veterinario Zootecnista, Maestría en Ciencias Biológicas – Universidad de Caldas
Tel. +57 317 471 4976; email: clavijomal@gmail.com

Personas a contactar

Si tiene cualquier pregunta acerca de este estudio o acerca de lo que debe hacer en caso de que sienta alguna molestia durante el estudio, puede comunicarse con el Dr. Alejandro Clavijo Maldonado al teléfono 3174714976.

Su médico veterinario, colaborador en esta investigación, estará disponible para responder cualquier pregunta adicional.

Nombre del Médico __ _____
Dirección del médico _ Centro Veterinario Mascotas
Teléfono del médico __881 5590 – 310831 6247_____

Terminación del estudio

Usted entiende que su participación en el estudio con su mascota es VOLUNTARIA. En cualquier momento usted puede retirar su consentimiento a participar en el estudio.

Autorización para uso de las muestras y datos obtenidos en este estudio

Se le solicita la autorización al participante para que las muestras y datos obtenidos en este estudio, puedan ser utilizados en otros estudios y laboratorios, previa Aprobación del Comité de Ética para la Experimentación con Animales (CEEA).

Aceptación

Obedeciendo a la Resolución 008430 del Ministerio de Salud Nacional.

SU FIRMA (O HUELLA DIGITAL) INDICA QUE USTED HA DECIDIDO PARTICIPAR VOLUNTARIAMENTE CON SU MASCOTA EN ESTE ESTUDIO HABIENDO LEIDO (O ESCUCHADO) LA INFORMACION ANTERIOR.

Fecha de firmado este documento: _____

Nombre de la paciente: _____

Nombre del propietario o tendedor: _____

Firma del propietario o tendedor: _____

CC. _____

Número de teléfono fijo: _____ Número de teléfono celular: _____

Dirección: _____

Nombre y firma del delegado del proyecto: _Alejandro Clavijo Maldonado_____

Número telefónico del delegado del proyecto: __314 471 4976_____

Anexo 4. Formato registro de paciente para toma de muestra y seguimiento.

Formato seguimiento paciente TMC-expresión génica - Control toma de muestras

	D	M	A		
				Consecutivo:	
Fecha:	_____/	_____/	_____	Nombre paciente:	
Ubicación:				HC paciente:	
MV-atención:				Tenedor/propietario:	
Centro Veterinario:				Núm Telefónico:	

Datos geneales del paciente

Especie				Raza	Tipo raza				Sexo	Edad	Peso aprox.	Color
C		F			Mestizo		Puro					

Dimensión del paciente						Condicion corporal								Dieta				
G		Me		P		Mi		1_3		4_5		6		7_9		A. Balanceado		Frecuencia 1
																C. casera		2
																Desperdicios		3
																Otro		4

Estado reproductivo

Entera	Si		No				No. Partos		Tto hormonal		
Esteilizada	Si		No		Fecha				Si		No
No. Celos			Último celo				Último parto		Cual:		

Información tumoral

Tipo de tumor (Dx presuntivo)	
Hiperplasia	
Neoplásia	

Diámetro **T**: tumor primario	
T0	No evidencia
T1	< 3 cm
T2	3-5 cm
T3	> 5 cm
T4	Carcinoma inflamatorio
a)	No fijo
b)	Fijo en piel
c)	Fijo en músculo

Estatus **N**: NL Regional	
N0	Sin evidencia Dx metástasis
N1	Con evidencia Dx metástasis

Metastasis: **M** a distancia	
M0	Sin metástasis a distancia
M1	Con metástasis a distancia

Grupo de estados clínicos			
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
IV	T1-T4	N0	M0
V	T1-T4	N1	M1

Hallazgos

Ubicación			
	L Izq		L Der
1	( )	1	( )
2	( )	2	( )
3	( )	3	( )
4	( )	4	( )
5	( )	5	( )

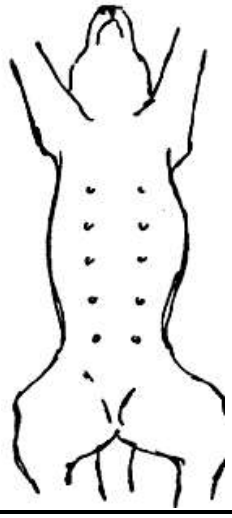
T			
	L Izq		L Der
1	[ ]	1	[ ]
2	[ ]	2	[ ]
3	[ ]	3	[ ]
4	[ ]	4	[ ]
5	[ ]	5	[ ]

N			
N0		N1	

M			
M0		M1	

Estado clínico			
I		III	
II		IV	

Vista ventral



Reg fotográfico

OBSERVACIONES:

	D	M	A
Fecha Dx inicial	_____/	_____/	_____
Dx final			
Tipo de intervenc			
Plan terapeutico			

Fecha de mortalidad	D	M	A
	_____/	_____/	_____

ID MUESTRA:

Exámenes y análisis de laboratorio anexo:

Anexo 5. Acta 3 de 2016. Aprobación del Comité de Ética para Experimentación con Animales (CEEA).

UNIVERSIDAD DE CALDAS
VICERRECTORÍA DE INVESTIGACIONES Y POSTGRADOS
COMITÉ DE ÉTICA PARA EXPERIMENTACIÓN CON ANIMALES (CEEA)

Manizales, 1 de junio de 2016

Doctor
ALEJANDRO CLAVIJO MALDONADO
Centro de Automatización Industrial
SENA Regional Caldas
Manizales

Asunto: aprobación de un proyecto de investigación (Código: 15061601)

Apreciado doctor Clavijo:

El CEEA reunido de manera extraordinaria el 31 de mayo de 2016, y tal como consta en el Acta 3 de 2016, aprobó la realización del proyecto: CÁNCER MAMARIO Y EXPRESIÓN DEL GEN HER-2 EN *Canis Lupus familiaris Linneaus*, 1758 (*Canidae*), en el Departamento de Caldas, Colombia. Este proyecto será presentado a la Convocatoria General de Investigaciones de la Universidad de Caldas.

Le deseamos éxitos en la realización de su estudio y en caso de que este sea aprobado en dicha u otra convocatoria, le solicitamos informar por escrito sobre el asunto a este comité.

Cordialmente,

JORGE U. CARMONA
Secretario Técnico

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Las referencias bibliográficas contenidas en este apartado, no se encuentran en los capítulos correspondientes a resultados.

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