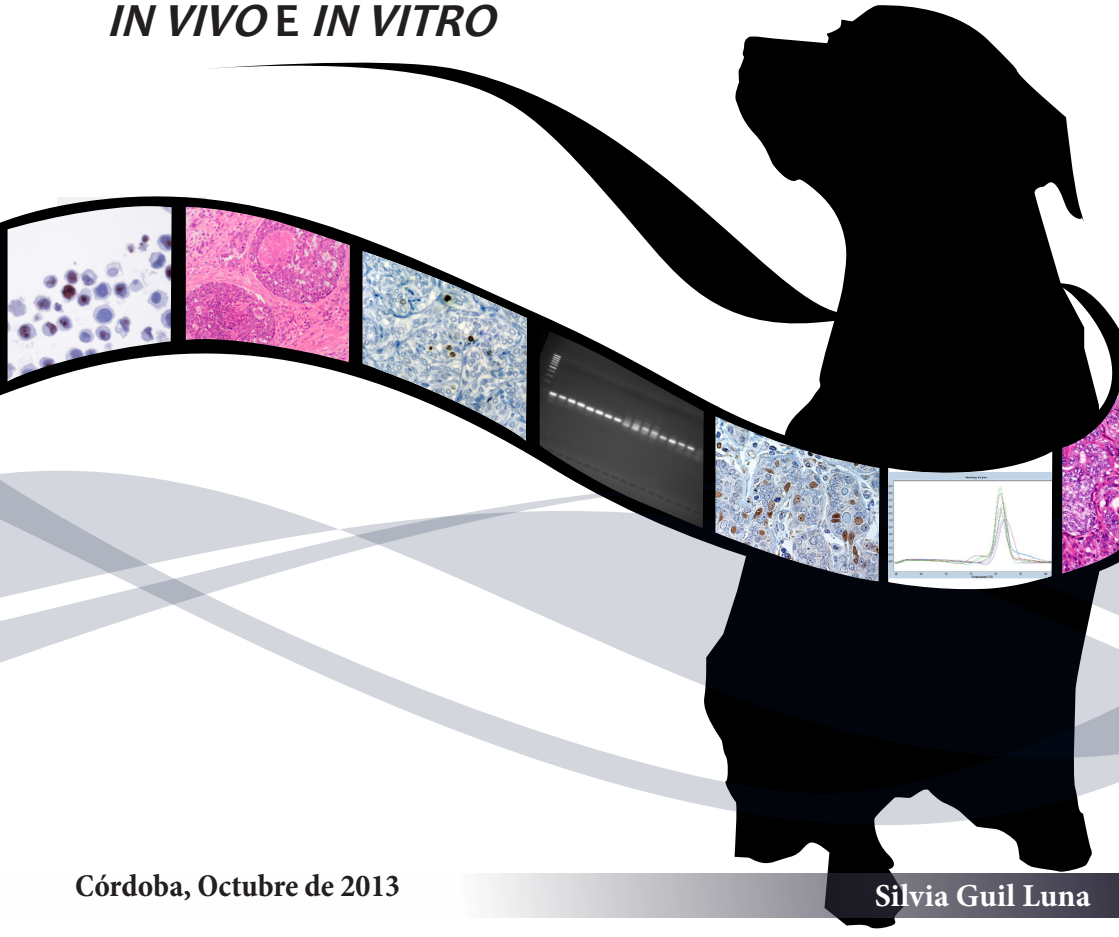
UNIVERSIDAD D CÓRDOBA

Departamento de Anatomía y
Anatomía Patológica Comparadas

EFECTOS DE LOS ANTIPROGESTÁGENOS SOBRE LA PROLIFERACIÓN DEL CARCINOMA DE MAMA CANINO. ESTUDIOS *IN VIVO* E *IN VITRO*



Córdoba, Octubre de 2013

Silvia Guil Luna

TITULO: *Efectos de los antiprogestágenos sobre la proliferación del carcinoma de mama canino. Estudios in vivo e in vitro*

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Efectos de los antiprogestágenos sobre la proliferación del carcinoma de mama canino. Estudios *in vivo* e *in vitro*.

Tesis Doctoral presentada por Silvia Guil Luna para optar al grado de Doctora.

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TÍTULO DE LA TESIS:
Efectos de los antiprogestágenos sobre la proliferación del carcinoma de mama canino. Estudios *in vivo* e *in vitro*.

DOCTORANDO/A: Silvia Guil Luna

INFORME RAZONADO DEL/DE LOS DIRECTORES DE LA TESIS

(Se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma)

Esta tesis doctoral se ha desarrollado durante un año de trabajo como contratada con cargo a proyecto más cuatro años de tutela académica como becaria FPU de la licenciada Silvia Guil Luna. Durante todo este tiempo, su dedicación y esfuerzo le han permitido realizar cuatro artículos científicos actualmente publicados o en revisión en revistas indexadas en el Journal Citation Reports que constituyen el cuerpo de la tesis doctoral, así como realizar una estancia en Zurich en la que desarrolló parte de los estudios reflejados en la primera publicación, una estancia en Copenhague durante la que realizó los estudios técnicos de la segunda y tercera publicaciones, y una estancia en Uppsala en la que desarrolló los estudios *in vitro* de la cuarta publicación.

El trabajo desarrollado sobre los efectos de los antiprogestágenos sobre la proliferación del carcinoma de mama canino novedoso en todos y cada uno de sus aspectos. Primero, por sus aportaciones a la posibilidad de uso del tratamiento endocrino en perras con cáncer de mama, y segundo porque se han utilizado métodos no descritos previamente en muestras de mama de la especie. Además, contribuye al conocimiento de la progresión del cáncer de mama en cualquier especie, incluida la humana. El primer aspecto se refleja en los efectos anti-proliferativos de los antagonistas del receptor de progesterona estudiados sobre las células tumorales con receptores de progesterona tanto *in vivo* como *in vitro*. Y el segundo, en la detección del ARNm del receptor de progesterona completo y de sus isoformas A y B en muestras de tumores de mama procesados rutinariamente para estudio histopatológico, algo sólo logrado hasta la fecha en la especie humana.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 2 de Septiembre de 2013

Firma de las directoras

Fdo: Juana Martín de las Mulas González-Albo

Fdo: Mª Yolanda Millán Ruiz

A mi familia,
padres, hermanos, Jaime y
en especial a Mara.

"El éxito de la vida no está en vencer
siempre sino en no rendirse nunca"

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1. Introducción y objetivos

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Los tumores de mama de la perra son los más frecuentes en las hembras de la especie canina. En Estados Unidos, su incidencia es de 198/100.000/año, pero allí la castración temprana, que tiene efecto protector sobre el desarrollo de estos tumores, es una práctica habitual. Esto no sucede en España, y además, se utilizan progestágenos sintéticos para controlar las manifestaciones del estro en la perra, productos que inducen la aparición de tumores de mama. Por todo ello, la incidencia de los tumores de mama de la perra en España es, muy probablemente, mayor que en Estados Unidos.

Algo menos de la mitad de los tumores de mama de perra tienen comportamiento biológico maligno y, en muchos de ellos, las células tumorales tienen receptores de progesterona. En la mujer, un elevado porcentaje de carcinomas de mama tiene receptores hormonales y en esos casos se utiliza el tratamiento endocrino de forma rutinaria como adyuvante o neoadyuvante a la cirugía porque la respuesta clínico-evolutiva es muy favorable pero en la perra no se utiliza este tipo de tratamiento.

La progesterona y los progestágenos sintéticos inducen la proliferación del epitelio glandular mamario mediante su unión al receptor de progesterona y se ha comprobado, tanto en estudios *in vivo* como *in vitro* de algunas especies, incluyendo la canina, que los antiprogestágenos,

compuestos que bloquean las acciones de los progestágenos naturales y sintéticos mediante su unión competitiva al receptor, bloquean esta acción. Sin embargo, en la perra no se han analizado los efectos de los antiprogestágenos sobre los tumores de mama ni *in vivo* ni *in vitro*.

La hipótesis de partida de este trabajo es que el bloqueo del receptor de progesterona disminuye la proliferación de las células neoplásicas del carcinoma de mama canino.

Para analizar la bondad de la hipótesis, los objetivos concretos planteados fueron los siguientes:

1. Estudiar los efectos *in vivo* del antiprogestágeno aglepristona sobre la proliferación de carcinomas de mama caninos con expresión inmunohistoquímica de receptores de progesterona.

2. Estudiar la expresión del receptor de progesterona completo y de sus isoformas A y B en muestras de tejido fijadas en formol e incluidas en parafina de carcinomas de mama caninos mediante RT-qPCR.

3. Estudiar *in vivo* la relación entre los efectos del antiprogestágeno aglepristona sobre la proliferación de carcinomas de mama caninos y la expresión de las isoformas A y B del receptor de progesterona analizada mediante RT-qPCR.

4. Estudiar los efectos *in vitro* de los antiprogestágenos mifepristona y onapristona sobre la proliferación de la línea celular de carcinoma de mama canino CMT-U27.

2. Revisión bibliográfica

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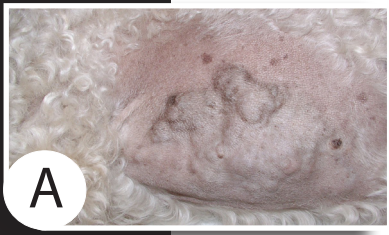
2.1. El carcinoma de mama canino

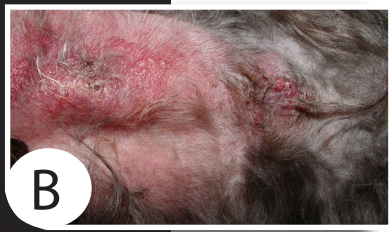
2.1.1. Características epidemiológicas y clínico-evolutivas

La importancia de los tumores mamarios caninos radica en su elevada incidencia. Para la mayoría de los autores representan el 50%, aproximadamente, de los tumores que padecen las hembras de la especie canina y su incidencia es de 200 por 100.000 perras en riesgo (Dorn y cols., 1968; Schneider y cols., 1970; Schneider y cols., 1976; Bostock y cols., 1986; Madewell y Theilen., 1987; Misdorp y cols., 1999; Arnesen y cols., 2001; Sorenmo y cols., 2013). Recientemente se han publicado datos que revelan diferencias geográficas en la incidencia de los tumores de mama de la perra y que parecen estar relacionados con la influencia que las hormonas ováricas tienen en el desarrollo de estos tumores. Así, la incidencia en el Reino Unido, donde es frecuente la ovariohisterectomía en edades tempranas, es de 205 casos por 100.000 perras (Dobson y cols., 2002) mientras que en Suecia la incidencia es de 111 casos por 10.000 perras ya que esta práctica quirúrgica es poco frecuente (Egenvall y cols., 2005). Desafortunadamente, en España no disponemos de datos sobre la incidencia de tumores mamarios en perras aunque es muy probable que las cifras sean cercanas a las de países como Suecia pues la ovariohisterectomía no es una práctica demandada de forma rutinaria y generalmente sólo se lleva a cabo en aquellas circunstancias en las que el veterinario la recomienda por motivos médicos.

Figura 1. Tumores de la mama canina.



Carcinoma de mama en una perra.



Carcinoma inflamatorio bilateral en una perra.

Fotos: Cortesía del Dr. Víctor Domingo Roa.

Los tumores de mama se presentan clínicamente como nódulos únicos o múltiples localizados dentro de la glándula mamaria (Figura 1A). La presentación como nódulos múltiples ocurre en más de la mitad de los casos, y pueden desarrollarse de manera simultánea o subsiguiente, asociados al pezón, o lo que es más frecuente, inmersos en el propio tejido mamario. La perra tiene 5 pares de glándulas mamarias, y todas pueden desarrollar uno o más tumores benignos o malignos. Entre el 65 y el 70% de los tumores de mama caninos ocurren en los pares 4 y 5, probablemente debido al mayor volumen de tejido glandular mamario que hay en estas glándulas (Sorenmo y cols., 2013).

Una excepción a esta forma de presentación clínica de los tumores de mama en la perra es el denominado carcinoma inflamatorio (Figura 1B) que es el más grave de todos ya que presenta un curso clínico fulminante y una supervivencia extremadamente baja (Pérez-Alenza y cols., 2001; Peña y cols., 2003a; Peña y cols., 2003b). Se observa una inflamación difusa que afecta a parte

de una cadena mamaria, a toda una cadena, o incluso a parte de las dos cadenas mamarias. La presencia de úlceras y el dolor son frecuentes. Además, puede haber edema en la extremidad o extremidades adyacentes. Este cuadro clínico en ocasiones se confunde con una mastitis y puede ser tratado erróneamente. En ocasiones también se describen coagulopatías en perras con carcinoma inflamatorio. (Susanek y cols., 1983; Pérez-Alenza y cols., 2001, Marconato y cols., 2009; Sorenmo y cols., 2013).

Los tumores malignos pueden metastatizar a los nódulos linfáticos regionales, por lo que la exploración clínica de una perra con tumores mamarios debe incluir siempre el examen de los nódulos linfáticos regionales. Dado que los tumores se localizan con mayor frecuencia en los pares 4 y 5, los nódulos linfáticos afectados con mayor frecuencia son los inguinales superficiales. Si éstos están aumentados de tamaño a la palpación, entonces se debe hacer un tacto rectal para explorar los ilíacos internos, ya que puede haber ocurrido diseminación hasta ellos, y radiografía o ecografía abdominal para evaluar el estado de los nódulos linfáticos sublumbares (Madewell y Theilen, 1987; Sorenmo y cols., 2013).

Además, los tumores malignos pueden desarrollar metástasis a distancia, siendo la pulmonar la más común, que se diagnostica mediante radiografía de tórax en un 25-50% de las perras con carcinoma de mama (Hedlund, 2007). También pueden ocurrir metástasis en hígado o riñón (Madewell y Theilen, 1987; Sorenmo y cols., 2013), incluso pudiendo

darse el caso de que la sintomatología de presentación sea la metástasis ósea, aunque no es frecuente (Cooley y Waters, 1998).

2.1.2. Factores de riesgo

Los principales factores de riesgo en el desarrollo del carcinoma de mama canino son los factores constitucionales (sexo, edad y raza) y los factores relacionados con la reproducción. Otros factores de riesgo más controvertidos o menos estudiados son la dieta, la exposición a radiaciones, la historia familiar y los antecedentes individuales de lesiones mamarias benignas o malignas.

Las neoplasias mamarias caninas son casi exclusivas de las hembras (Brodey y cols., 1983; Madewell y Theilen, 1987, Saba y cols., 2007; Bearss y cols., 2011), y la edad media de presentación es de 10 - 11 años para los tumores malignos y algo menor para los benignos (Schneider, 1970; Bostock, 1986; Madewell y Theilen, 1987; Sorenmo y cols., 2013). Se ha descrito una mayor incidencia en algunas razas puras de pequeño tamaño como Caniche, Chihuahua, Yorkshire terrier, Maltés y Cocker spaniel (Sorenmo y cols., 2013) pero algunas razas grandes, como Boxer, Pastor alemán o Springer spaniel inglés, también tienen mayor riesgo de desarrollar este tipo de tumores (Goldschmidt y cols., 2001; Egenvall y cols., 2005; Itoh y cols., 2005). Sin embargo, el factor de riesgo más importante es la exposición del parénquima glandular mamario a las hormonas ováricas, estrógenos y progesterona (Schneider y cols., 1969; Giles y cols., 1978; Misdorp, 1991; Rutteman, 1992; Støvring y cols., 1997).

Se ha comprobado que la ovariectomía temprana tiene un intenso efecto protector sobre el desarrollo de tumores de mama tanto benignos como malignos y que este efecto protector disminuye con el número de ciclos que haya tenido el animal antes de la castración o incluso desaparece si la ovariectomía tiene lugar después de los 4 años de edad (Schneider y cols., 1969; Misdorp y cols., 1988). También se ha observado que la administración prolongada de hormonas ováricas, de forma clínica o experimental, aumenta el riesgo de desarrollar tumores mamarios. Así,

- La administración prolongada de acetato de medroxiprogesterona y de proligestona a perras jóvenes aumenta el riesgo de desarrollar tumores y el efecto es dependiente de la dosis y de la regularidad o irregularidad del tratamiento (Misdorp, 1991; Støvring y cols., 1997).
- También se ha observado que las dosis bajas de progestágenos aumentan el riesgo de desarrollar tumores benignos mientras que la combinación de estrógenos y progestágenos aumenta el riesgo de desarrollar tumores malignos (Geil y cols., 1977; Giles y cols., 1978; Kwapien y cols., 1980; Concannon y cols., 1981; Selman y cols., 1995).
- En estudios de toxicidad en perras Beagle se ha observado que los progestágenos provocan lesiones en las mamas, pero los estrógenos no (Rutteman, 1992).
- En los tratamientos con progestágenos sintéticos y con progesterona se ha observado, 1º, que el tratamiento

produce nódulos mamarios múltiples; 2º, que las lesiones son reversibles; 3º, que el tipo de lesión desarrollada es dependiente de la dosis, puesto que las dosis bajas producen hiperplasias y displasias y solo las muy altas producen tumores; y 4º, que los tumores que se desarrollan con más frecuencia son benignos (Giles y cols., 1978; Misdorp, 1991; Misdorp, 2002).

El riesgo de desarrollar tumores malignos aumenta después de la administración experimental de estrógenos combinados con progestágenos a dosis altas o si las drogas usadas tienen una actividad combinada estrógenos - progesterona (Giles y cols., 1978; Kwapien y cols., 1980). Así, la administración prolongada de dosis altas (125 x dosis humana) de 19-Nortestosterona indujo la aparición de cáncer de mama en el 40% de las perras enteras así tratadas (Misdorp, 1991). Por el contrario, las combinaciones estrógenos - progesterona a dosis bajas parecen ofrecer cierto grado de protección (Misdorp, 1991).

Finalmente, las dietas ricas en grasa y la obesidad parecen aumentar el riesgo de desarrollar tumores de mama en las perras, sobre todo cuando se administran en el primer año de vida (Sonnenschein y cols., 1991; Pérez-Alenza y cols., 1998; Pérez-Alenza y cols., 2000), mientras que la exposición a radiaciones no parece aumentar la incidencia de tumores mamarios, aunque sí su aparición a edades

más tempranas (Andersen y Rosenblatt, 1969; Moulton y cols., 1970; Moulton y cols., 1986; Benjamin y cols., 1999).

En medicina humana, la existencia de algunos tipos de displasia como la hiperplasia atípica y del carcinoma in situ (CIS) se considera un factor importante de riesgo para padecer posteriormente un carcinoma infiltrante (Page y Dupont, 1990). En perras también se han descrito este tipo de patologías mamarias y, al igual que sucede en la mujer, se ha sugerido que podrían ser un factor de riesgo sobre la incidencia ulterior de los tumores malignos en el mismo animal (Moulton, 1990; Antuofermo y cols., 2007; Mouser y cols., 2010; Sorenmo y cols., 2013).

Las alteraciones genéticas específicas involucradas en el desarrollo de los carcinomas de mama caninos, y su grado de participación en dicho desarrollo, son muy poco conocidas en el momento actual, aunque están realizándose estudios cuyos resultados están empezando a reflejarse en la literatura. Así, el estudio de genotipos y los análisis de expresión génica y proteica indican que hay varios genes involucrados en el desarrollo de los tumores de mama caninos incluyendo el c-erbB-2 (Ahern y cols., 1996; Rungsipipat y cols., 1999; Martín de las Mulas y cols., 2003; Gama y cols., 2008), el gen supresor de tumores p53 (Chu y cols., 1998; Veldhoen y cols., 1999), los genes inhibidores de quinasas p21 y p27 (Klopfleisch y cols., 2009) y los genes BRCA1 y BRCA2 (Rivera y cols., 2009; 2011). Un reciente estudio ha relacionado la presencia de mutaciones en el gen

del receptor de estrógenos α con el riesgo de desarrollar tumores de mama caninos (Borge y cols., 2013).

2.1.3. Diagnóstico, pronóstico y tratamiento

El método único y definitivo de diagnóstico de un tumor de mama es el estudio anatomopatológico, y el tipo de biopsia que se practica es la biopsia excisional. La citología por punción-aspiración con aguja fina de un tumor mamario es útil para el diagnóstico del carcinoma inflamatorio porque ayuda a descartar procesos benignos como mastitis (Allen y cols., 1986; Madewell y Theilen, 1987; Hellmén y Lindgren, 1989; Sorenmo y cols., 2013). Sin embargo, esta técnica no es suficiente para confirmar la enfermedad ya que no revela la afectación linfática característica del carcinoma inflamatorio (Pérez-Alenza y cols., 2001). La correlación entre la citología previa y la histopatología posterior de tumores de la mama canina ha demostrado ser de entre el 67.5% y el 93% con sensibilidad (88%) y especificidad (96%) altas (Rollón, 2003; Sorenmo y cols., 2013).

Los criterios que se utilizan para clasificar las neoplasias de la mama canina son similares a los utilizados en todos los tipos de neoplasias: comportamiento biológico y morfología, pudiendo ser ésta última descriptiva o histogenética. La clasificación que se ha usado comúnmente en la mayoría de los laboratorios de anatomía patológica veterinaria y en los trabajos científicos publicados es la propuesta por la Organización Mundial de la Salud (OMS) y el Instituto de la Fuerzas Armadas de los Estados Unidos de Norteamérica (Misdorp y cols., 1999). Sin embargo, esta

clasificación presenta ciertas limitaciones ya que hay tipos histológicos que no están incluidos o no hacen distinción entre algunos tipos histológicos de tumores. Goldschmidt y colaboradores propusieron en 2011 una nueva clasificación de los tumores de la mama canina que pretende soslayar estas limitaciones.

El pronóstico del carcinoma de mama depende de una serie de características clínicas e histopatológicas. Las características clínicas con valor pronóstico establecido son el tamaño tumoral, la presencia de metástasis a nódulos linfáticos regionales en el momento del diagnóstico y el estadio clínico de la enfermedad neoplásica, y las características histopatológicas con valor pronóstico son el grado histológico de malignidad, la invasión vascular sanguínea o linfática, el crecimiento infiltrante en tejidos vecinos, la presencia de metástasis en nódulos linfáticos regionales y el tipo histológico de tumor (Sorenmo y cols., 2013). Algunos estudios indican que la expresión IHQ de receptores de estrógenos (RE) y receptores de progesterona (RP) son factores pronósticos favorables aunque no siempre se haya podido demostrar su naturaleza independiente (Nieto y cols., 2000; Martín de las Mulas y cols., 2005; Chang y cols., 2009).

Cuando un animal tiene más de un tumor, el pronóstico lo condiciona el más agresivo, y se debe seleccionar el de mayor tamaño o el que tiene una histopatología más agresiva (Sorenmo y cols., 2013). En muchos casos, el más agresivo histológicamente es el de mayor tamaño.

Exceptuando el carcinoma inflamatorio de mama, la cirugía es, a día de hoy, la terapia de elección del carcinoma de mama en la perra. Sin embargo, no existe una guía consensuada en la que se establezcan los criterios de selección que determinen el tipo de intervención a realizar según la presentación del tumor (Sorenmo y cols., 2013). En un estudio clínico prospectivo de 144 animales en el que se comparaba el tipo de cirugía, mastectomía simple frente a mastectomía de la cadena mamaria, no se encontraron diferencias ni en la tasa de supervivencia ni en la tasa de recidivas (Marconato y cols., 2009). Los argumentos a favor de cirugías más agresivas se basan en que este es, probablemente, el procedimiento más eficaz para eliminar la totalidad del tumor. Los argumentos en contra se basan, fundamentalmente, en que aproximadamente el 50% de los tumores mamarios caninos tienen un comportamiento biológico "benigno" en el que siempre es posible reintervenir al animal si el estudio histopatológico de la pieza extirpada en la primera cirugía así lo recomienda, mientras que las cirugías más agresivas aumentan la morbilidad, el tiempo de recuperación y los costes de tratamiento (Sorenmo y cols., 2013).

Básicamente, las modalidades de tratamiento sistémico del carcinoma de mama canino son dos, la quimioterapia y la terapia endocrina. El tratamiento sistémico más comúnmente utilizado es la quimioterapia adyuvante a la cirugía aún a pesar de que su eficacia ha sido comprobada en muy pocos ensayos clínicos (Sorenmo y cols., 2013). De hecho, se recomienda y administra quimioterapia después de

la cirugía a animales con tumores de alto riesgo utilizando protocolos muy variados que incluyen, entre otros, la doxorrubicina (30 mg/m2, cada 21 días), la mitoxantrona (5-6 mg/m2, cada 21 días), la gencitabina (800 mg/m2 semanal durante 4 semanas), la ciclofosfamida y el 5-fluouracilo. Uno de los trabajos publicados mostró un aumento significativo de la supervivencia en un grupo de animales con carcinomas en estadio clínico III/IV sometidos a un tratamiento con 5-fluorouracilo (150 mg/m2 intravenoso semanalmente) y ciclofosfamida (100 mg/m2 intravenosa semanalmente) (Karayannopoulou y cols., 2001). Ahora bien, en este trabajo el número de animales era pequeño (n = 16) y los grupos no fueron diseñados aleatoriamente. Más recientemente, un ensayo clínico con perras con carcinomas de mama de grados histológicos 2 y 3 mostró que la desmopresina, un análogo sintético de la hormona antidiurética vasopresina, prolongaba el tiempo de vida libre del animal tras la cirugía (Hermo y cols., 2011).

El tratamiento endocrino del cáncer de mama puede ser ablativo o aditivo. El tratamiento ablativo es la castración quirúrgica practicada en el momento de la cirugía del tumores o tumores mamarios, o bien la castración química postquirúrgica. El único tipo de tratamiento endocrino que se practica hoy día en los tumores de mama caninos es el ablativo quirúrgico, aunque no se usa de manera uniforme porque el posible efecto protector de la ovariectomía practicada en el momento de la cirugía es muy controvertido. Así, mientras que algunos estudios afirman que tiene efecto protector (Sorenmo y cols., 2000; Chang y cols., 2005)

otros lo niegan (Fowler y cols., 1974; MacEwen y cols., 1985; Yamagami y cols., 1996; Morris y cols., 1998; Philibert y cols., 2003).

A día de hoy, no está aclarado si realmente la ovariohisterectomía practicada después del periodo de protección de los dos primeros años de vida tiene algún beneficio en las perras con cáncer de mama. Hay dos estudios publicados que demostraron mejoras en la tasa de supervivencia. En uno de ellos la supervivencia de las perras con tumores de mama sometidas a una ovariectomía dentro de los dos años previos a la resección del tumor o en el momento de la mastectomía era mayor que en las perras que permanecían intactas (Sorenmo y cols., 2000). En el otro estudio también encontraron mayor supervivencia postquirúrgica cuando la ovariectomía se realizaba en el momento de la mastectomía (Chang y cols., 2005). Por el contrario, en un estudio realizado con 154 perras tratadas con mastectomía y ovariectomía no se observó ninguna ventaja en la supervivencia cuando se compararon sus resultados con los de otros trabajos en los que las perras eran sometidas únicamente a mastectomía (Fowler y cols., 1974). Otros estudios retrospectivos más recientes no demuestran mejora alguna ni para el tumor ni para la supervivencia (Yamagami y cols., 1996; Morris y cols., 1998). Es interesante destacar que en perras con tumores de mama benignos se ha observado que la ovariectomía practicada en el momento de la cirugía disminuye el riesgo de aparición de nuevos tumores en el 50% de los casos (Kristiansen y cols., 2013).

El tratamiento ablativo químico es útil para inhibir de forma reversible el eje hipotálamo – hipófisis – ovario. Para ello, se utilizan agonistas de la hormona liberadora de gonadotropina (GnRH) a dosis sostenidas en el tiempo. La goserelina, fármaco agonista de GnRH, se ha usado en perras con carcinoma de mama cada 21 días durante 12 meses y se ha observado una reducción significativa del tamaño del tumor y un aumento de la supervivencia a los 2 años en el 88% de los animales (Lombardi y cols., 1999).

En la perra con cáncer de mama no se utiliza el tratamiento endocrino aditivo con antiestrógenos o antiprogestágenos como adyuvante a la cirugía ya que el uso de antiestrógenos está desaconsejado debido a sus efectos agonistas en el aparato genital (Morris y cols., 1993; Hoffmann y Schuler, 2000) y el uso de antiprogestágenos no se ha ensayado.

2.2. El receptor de progesterona

2.2.1. Estructura, regulación y función

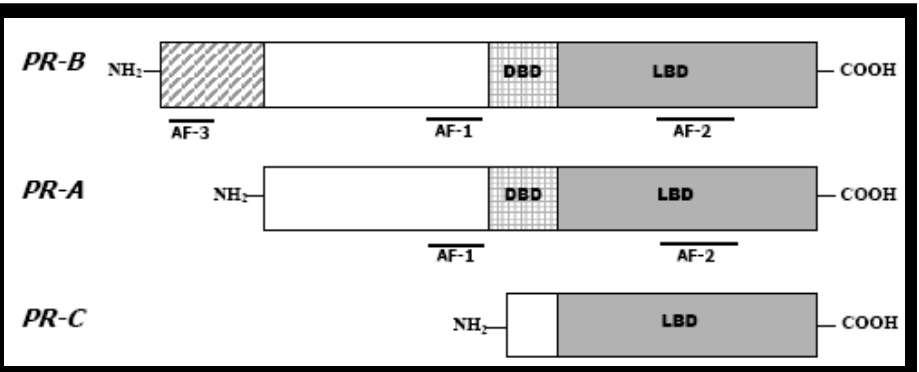
El RP, perteneciente a la familia de receptores esteroideos de los receptores nucleares, media la acción de la hormona progesterona, un ligando clave en el desarrollo normal de la glándula mamaria. Una vez este ligando natural (progesterona) o sintético (progestágenos) queda unido al RP, éste experimenta un cambio conformacional que le permite interaccionar con el ADN y regular la transcripción de ciertos genes implicados en una variedad de funciones tales como el crecimiento celular, la apoptosis y el metabolismo lipídico y esteroideo (Li y O´Malley, 2003).

En la especie humana, el RP tiene dos isoformas, isoforma A (RPA) e isoforma B (RPB) que se expresan a partir de un mismo gen (11at q22-23) pero que tienen diferentes promotores y sitios internos para el inicio de la traducción (Jacobsen y cols., 2002). Existe una tercera isoforma, la isoforma C (RPC), de 60 kDa, situada en la región N terminal del RP que ha sido poco estudiada hasta el momento y parece carecer de actividad transcripcional clásica (Wei y cols., 1997; Condon y cols., 2006).

Las isoformas RPA y RPB son idénticas ya que ambas contienen el dominio de unión al ligando (LBD) en el extremo C terminal, que es la región más constante del receptor, y una región de unión al ADN (DBD) centralmente localizada (Figura 2). Sin embargo, se diferencian en que a RPA le faltan 164 aminoácidos en el extremo amino (Figura 2). Los dominios LBD y DBD están unidos por 50 aminoácidos de secuencia bisagra (Kastner y cols., 1990). Las funciones de activación transcripcional están localizadas en el extremo-N de DBD (AF1) y dentro del LBD (AF2) en ambas isoformas mientras que la tercera función de activación, AF3, es única para RPB.

Es bien conocido que la activación del RE mediante la unión de su ligando natural, los estrógenos, induce la expresión génica del RP (Kastner y cols., 1990; Graham y Clarke, 1997; Hewitt y cols., 2000) pero los mecanismos de regulación de las isoformas del RP son menos conocidos. En estudios llevados a cabo con líneas celulares de cáncer de mama humano se han obtenido resultados contradictorios. Así, en la

Figura 2.
Estructura de las isoformas B, A y C del receptor de progesterona humano.



PR-B: isoforma B; PR-A: isoforma A; PR-C isoforma C; DBD: Dominio de unión al ADN, LBD: Dominio de unión al ligando, AF1-AF3: Dominios de activación

(Modificada de Rekawiecki y cols., 2008)

línea celular T47D se observó que los estrógenos estimulaban el RPB y que el tratamiento con progesterona disminuía la expresión tanto de RPA como de RPB (Graham y cols., 1995). Más tarde, un estudio realizado por Vienonen y colaboradores en 2002 demostró que los estrógenos podían regular al alza ambas isoformas en células T47D, RPA en la línea celular MCF-7 y RPB en la línea ZR-75-1.

A pesar de su alto grado de homología, RPA y RPB presentan distintas actividades transcripcionales. Así, RPB es, en general, un activador más fuerte que RPA, debido en parte a la existencia del tercer dominio de activación AF3 (Sartorious y cols., 1994). Por otro lado, RPA puede funcionar como un represor transdominante dependiente de ligando de

otros receptores esteroideos incluyendo RPB y RE. Además, estudios por *microarray* han demostrado que las dos isoformas regulan un conjunto de genes diferentes (Richer y cols., 2002; Jacobsen y cols., 2002).

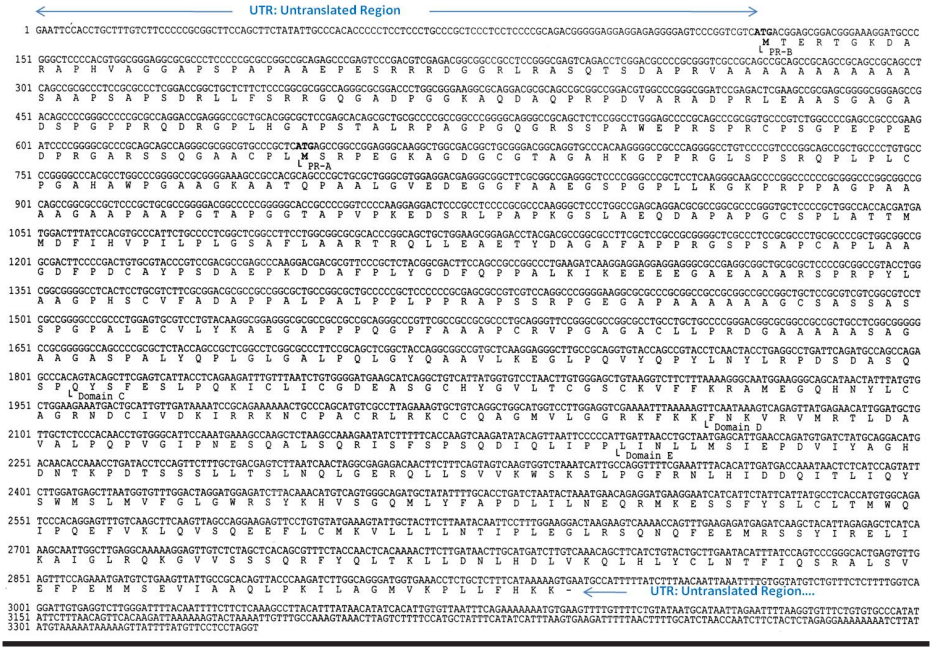
El gen del RP canino se secuenció y clonó por primera vez en al año 2000 por Lantinga van-Leeuwen y colaboradores. Las isoformas A y B del RP canino se han identificado en un número muy limitado de muestras de tejido uterino normal y de glándula mamaria normal y neoplásica mediante *Western blot* (Gracanin y cols., 2012a). Estas isoformas son altamente coincidentes con las ya descritas en la especie humana y también están codificadas por el mismo gen (Lantinga van-Leeuwen y cols., 2000) (Figura 3).

Además, el análisis de secuencia de nucleótidos ha demostrado que la isoforma B del RP canino presenta mutaciones en el dominio AF3 que condicionan que tenga un potencial transcripcional más limitado que en el RP humano y felino (Gracanin y cols., 2012b). Hasta la fecha no se han publicado estudios de expresión de las isoformas A y B del RP canino en tumores de mama mediante otros métodos de estudio.

2.2.2. Métodos de estudio *in vivo* e *in vitro*

Los primeros estudios de detección de receptores de hormonas esteroideas en los tumores espontáneos de la mama canina datan de los años 80 del siglo pasado. Entonces se utilizaban los métodos bioquímicos basados en la unión del ligando marcado radiactivamente

Figura 3.
Secuencia de nucléotidos del gen del receptor de progesterona canino y sus aminoácidos correspondientes y codones de iniciación de la traducción (ATG) para las isoformas A y B del receptor.



(Modificada de Lantinga van-Leeuwen y cols., 2000).

(estrógenos o progesterona) al receptor correspondiente. El más utilizado, al igual que en la especie humana, fue el método del "dextran-coated charcoal" o DCC (Baulieu & Mester, 1989). Desde entonces y hasta finales de los años 90 todos los datos sobre la expresión de RE y RP en los tumores de la mama canina se basaban en análisis con DCC (Hamilton y cols., 1977; MacEwen y cols., 1982; Mialot y cols., 1982a; Mialot y cols., 1982b; Elling y Ungemach, 1983; Ungemach, 1983; Martin y cols., 1984; Parodi y cols., 1984; Monson y cols., 1987; Rutteman

y cols., 1988; Donnay y cols., 1989; Rutteman, 1990; Donnay y cols., 1993; Donnay y cols., 1995).

Estos estudios en realidad eran muy escasos comparados con los que se hicieron en la mujer porque el método del DCC era muy caro y no era de fácil acceso a las Facultades de Veterinaria. De hecho, ni siquiera estaba disponible no ya en las Facultades de Veterinaria sino en muchos de los hospitales de la Seguridad Social en España. Además, requería del uso de muestras frescas y detectaba solamente los receptores no ocupados por su ligando natural tanto del tejido tumoral como del tejido adyacente no tumoral. El desarrollo de anticuerpos monoclonales altamente específicos frente a las proteínas tanto del RE como del RP humanos dieron paso al uso generalizado de las técnicas IHQ de detección de los receptores hormonales basadas en la unión inmunológica entre el receptor y los anticuerpos específicos desarrollados frente a él (Greene y cols., 1980; 1988; al Saati y cols., 1993; Goussard, 1998). Los métodos IHQ presentan importantes ventajas sobre los métodos bioquímicos, destacando entre ellas la posibilidad de uso en muestras de tejido procesadas rutinariamente para estudio histopatológico y la identificación precisa del receptor en la muestra de tejido con independencia de su estado de ocupación (De Mascarel y cols., 1995; MacGrogan y cols., 1996; Allred y cols., 1998).

A partir de los años noventa, varios autores estandarizaron las técnicas IHQ de detección de receptores hormonales en muestras

de tejido mamario canino utilizando los anticuerpos comerciales que habían sido desarrollados frente a los antígenos humanos ya que no había anticuerpos específicos frente a los receptores caninos disponibles comercialmente. El RP de la especie canina presenta un alto nivel de homología en la secuencia de aminoácidos con el RP humano, y particularmente en la región C-terminal (Figura 4). Por este motivo la mayoría de los anticuerpos disponibles comercialmente frente al RP humano son útiles para la detección del RP canino utilizando técnicas basadas en la reacción antígeno – anticuerpo.

Los diferentes autores obtuvieron cifras de expresión de RP dispares, tanto entre los tumores benignos (del 50% al 100%) como entre los tumores malignos (del 31.2% al 100%) (Tabla 1). Esta disparidad también se observó en los estudios de expresión de los RE (Manzel y cols., 1995; Graham y cols., 1999; Geraldes y cols., 2000; Nieto y cols., 2000; Martín de las Mulas y cols., 2005; Chang y cols., 2009).

Las discrepancias en las cifras de expresión IHQ de RP (y de RE) pueden ser debidas, al menos en parte, a diferencias en las poblaciones objeto de estudio (tamaño tumoral, estado de castración, tipos histológicos de tumor) y a razones de tipo técnico como los anticuerpos o los métodos de desenmascaramiento antigénico empleados, pero la causa más importante es probablemente la falta de un umbral de positividad basado en datos biológicos.

Figura 4. Homologías entre la secuencia de aminoácidos del RP humano y el RP canino.

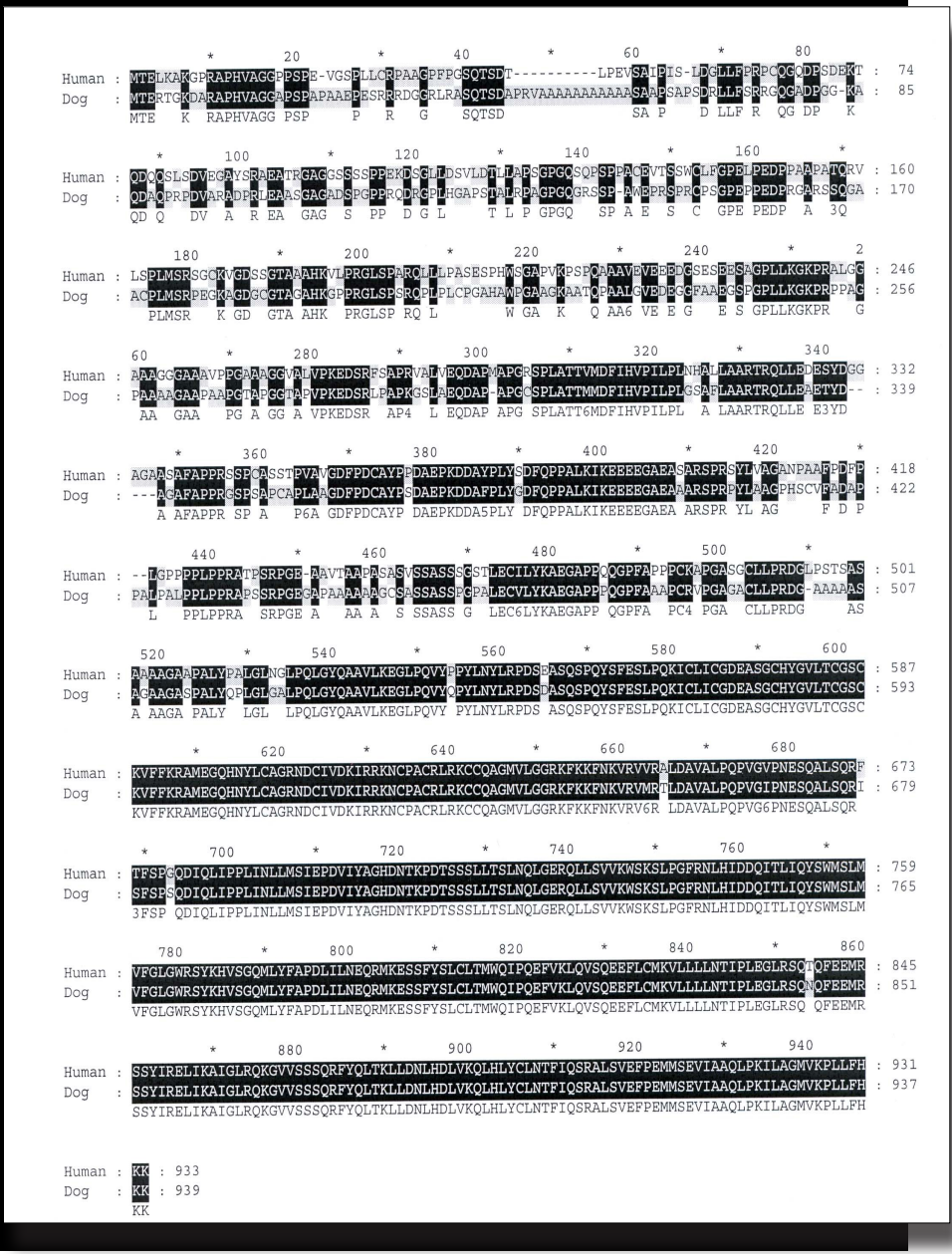


Tabla 1. Expresión de RP en tumores de mama caninos utilizando técnicas inmunohistoquímicas.

Autores	Tumores benignos (%)	Tumores malignos (%)
Manzel y cols., 1995	No estudiados	33
Geraldes y cols., 2000	100	60
Martín de las Mulas y cols., 2005	92	66
Chang y cols., 2009	100	71.9
Toniti et al., 2009	52	39
Millanta y cols., 2010	No estudiados	52
Mouser y cols., 2010	No estudiados	100
Sassi y cols., 2010	No estudiados	77
Dolka y cols., 2011	No estudiados	69
Beha y cols., 2012	No estudiados	50
Im y cols., 2013	No estudiados	58

En los años 80, las nuevas técnicas IHQ de detección de receptores hormonales en muestras de tejido fijadas en formol e incluidas en parafina se validaban por comparación con los resultados obtenidos en muestras de los mismos tumores mediante el método DCC siguiendo las normas del "National Institutes of Health Consensus Conference on estrogen

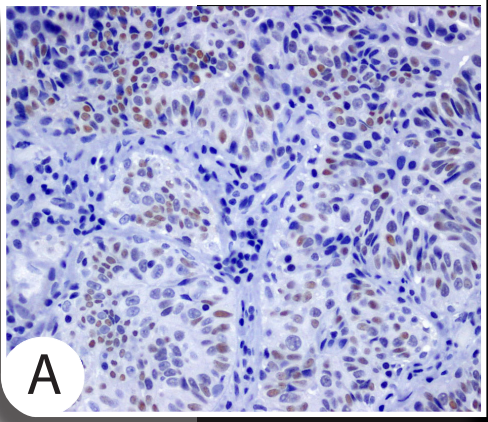
receptors in breast cancer" de 1979 (McCarty y cols., 1985). Este tipo de validación se llevó a cabo en un estudio con muestras de tejido canino para la detección de RE y en dos estudios con muestras de tejido felino para la detección de RE y RP exclusivamente (Graham y cols., 1999; Martín de las Mulas y cols., 2000a; Martín de las Mulas y cols., 2002).

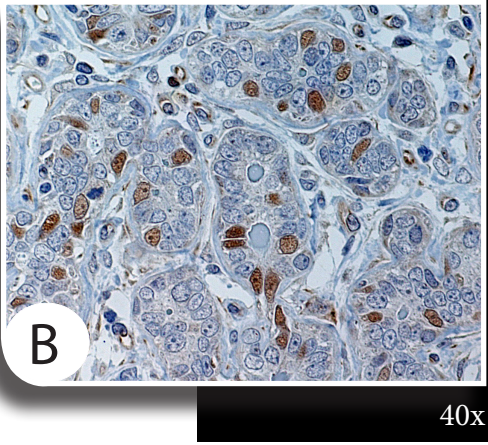
Sin embargo, la validación más importante de una técnica IHQ de detección de receptores hormonales es la validación biológica. La validación biológica consiste en comprobar si la expresión IHQ del receptor correspondiente se asocia a un comportamiento clínico determinado o a una respuesta favorable al tratamiento endocrino adyuvante a la cirugía (Fitzgibbons y cols., 2010). En la mujer, se ha comprobado que la expresión de RE y/o de RP en el carcinoma de mama es un factor predictivo de pronóstico favorable y de respuesta favorable al tratamiento endocrino adyuvante a la cirugía (Baker y cols., 1990; Allred y cols., 1998; Fitzgibbons y cols., 2010). Además, se ha observado que existe respuesta al tratamiento endocrino cuando el 1% de las células tumorales expresan receptores hormonales (Fitzgibbons y cols., 2010; Hammond y cols., 2010). De esta manera existe un umbral biológico de positividad que permite homogeneizar los resultados obtenidos por los diferentes laboratorios.

En la perra no se ha podido establecer todavía un umbral biológico de positividad porque, primero, los pocos estudios que han analizado el valor pronóstico de la expresión de RE y de RP han arrojado resultados

contradictorios (Nieto y cols., 2000; Martín de las Mulas y cols., 2005; Millanta y cols., 2005; Chang y cols., 2009) y segundo, porque no se ha analizado la correlación entre la expresión del RP y el tratamiento endocrino adyuvante a la cirugía.

Figura 5. Expresión de RE alfa (A) y RP (B) en tumores de mama caninos utilizando técnicas inmunohistoquímicas





Hoy día se siguen utilizando los métodos IHQ de detección del RP, al igual que del RE (Figura 5), para estudios clínicos y estudios de investigación aún a pesar de los inconvenientes que supone la ausencia de umbrales biológicos pero hay otros métodos que son más objetivos y que no se utilizan en medicina veterinaria para analizar el RP y sus isoformas. Entre ellos destaca la reacción cuantitativa en cadena de la polimerasa con transcriptasa inversa (RT-qPCR), que

permite cuantificar la expresión del RP y sus isoformas de una manera más precisa y fiable.

En medicina humana existen numerosos estudios que prueban la utilidad de esta técnica para la detección de receptores hormonales esteroideos en muestras de cáncer de mama habiéndose observado una elevada concordancia entre los resultados de RT-qPCR e IHQ (Badve y cols., 2008; Iverson y cols., 2009; Oda y cols., 2010). Igualmente, hay numerosos estudios de expresión del RP a nivel de ARNm en útero, vagina, hipotálamo e hipófisis de ratón, rata, cerdo, gallina y gato, entre otros (Iwai y cols., 1991; Tseng L y Zhu HH, 1997; Kurita y cols., 2000; Fang y cols., 2002; Ocón-Grove y cols., 2007; Gracanin y cols., 2012a;2012b). Las isoformas A y B del RP se han estudiado también mediante métodos bioquímicos como el *Western blot* y la inmunoprecipitación en la especie humana, así como por técnicas IHQ. Sin embargo, el uso de estas técnicas no está muy extendido debido a los problemas de especificidad que al parecer presentan los anticuerpos anti-RPA y anti-RPB disponibles hasta el momento (Mote y cols., 2001). Por eso, la RT-qPCR se considera en la actualidad la técnica más sensible y fiable para la detección de las isoformas del RP humano (Kariagina y cols., 2008). En el perro, no se han publicado estudios sobre la detección del RP mediante RT-qPCR.

2.2.3. Participación en el carcinoma de mama

Los estudios de detección de RP en el carcinoma de mama canino con técnicas IHQ han mostrado que, en general, la expresión de RP es

más frecuente que la expresión de RE (Geraldes y cols., 2000; Martín de las Mulas y cols., 2005; Chang y cols., 2009), hallazgo que sugiere que la progesterona podría jugar un papel más importante que los estrógenos en el desarrollo de la enfermedad. La elevada expresión de RP junto con las numerosas evidencias epidemiológicas y clínicas del papel proliferativo de la progesterona en los tumores de mama de la perra (Misdorp, 1991; Rutteman, 1992; Selman y cols., 1995; Støvring y cols., 1997) abre la posibilidad del uso del tratamiento sistémico del carcinoma de mama mediante antiprogestágenos.

Ya en los años 60 se sospechaba que la exposición prolongada a elevadas concentraciones de progesterona durante la larga fase lútea del ciclo estral era clave en el desarrollo de los tumores de mama de la perra (Evans y cols., 1969). En el año 1980, Concannon y colaboradores observaron que los progestágenos sintéticos inducían un síndrome de exceso de hormona de crecimiento (GH) en la perra que podía llegar a producir acromegalia, y un año después publicaron que los mismos compuestos producían tumores de mama (Concannon y cols., 1981). Siempre se creyó que la GH procedía de la adenohipófisis, aunque los estudios de supresión no parecían confirmarlo, y 13 años después se demostró que la hormona de crecimiento procedía del epitelio tumoral de la mama (Selman y cols., 1994).

También en 1980 se describió el desarrollo de carcinomas de mama en perras tras la administración de dosis elevadas de progestágenos

(Kwapien y cols., 1980). Y más recientemente, que el tratamiento de perras ovariectomizadas con progestágenos durante 6-12 meses indujo la aparición de hiperplasias y / o tumores benignos de mama (Selman y cols., 1994; Bhatti y cols., 2007). Finalmente, estudios recientes de expresión génica indican que los progestágenos tienen marcados efectos proliferativos en la hiperplasia y el carcinoma de mama canino (Rao y cols., 2009).

En otras especies también hay datos que indican que la progesterona ejerce acciones proliferativas sobre el epitelio glandular mamario normal y neoplásico. En estudios realizados en ratones *knockout* para el RP se comprobó que el RP es necesario para el desarrollo normal de la glándula mamaria (Mulac-Jericevic y cols., 2004). Así, el RE promueve el crecimiento temprano de los conductos lácteos mientras que tanto el RE como el RP son responsables del desarrollo de las terminaciones mamarias o alveolos localizados en los extremos de los conductos que se convertirán en las estructuras productoras de leche en la glándula mamaria (Lange y cols., 2009).

En estudios *in vitro* se ha observado que la progesterona estimula la proliferación de líneas celulares de carcinoma de mama humano con RP (T47D y MCF-7) (Moore, 2004). Además, en estudios experimentales se ha observado que el acetato de medroxiprogesterona induce la aparición de carcinomas de mama con RP en ratonas BALB/c (Helguero y cols., 2003). Por último, en estudios clínicos se ha observado que en la

mujer menopáusica que recibe terapia hormonal sustitutiva combinada (estrógenos más progestágenos) el riesgo de desarrollar cáncer de mama es mucho mayor (8% al año) que si es solo estrogénica (1% al año) (Moore, 2004).

Numerosos datos indican que los progestágenos ejercen sus acciones proliferativas a través del RP. Así, en estudios *in vitro* se ha observado que los antiprogestágenos como el RU486 (*mifepristona*) y el ZK299 (*onapristona*) inhiben la proliferación de líneas celulares T47D y MCF-7 de carcinoma de mama humano y que dicha inhibición está mediada por los RP (Schneider y cols., 1990; Horwitz, 1992).

En estudios experimentales se ha observado que tanto los carcinomas de mama con RP inducidos por el acetato de medroxiprogesterona en ratonas BALB/c como sus metástasis pulmonares y axilares regresan tras el tratamiento con antiprogestágenos (Vanzulli y cols., 2005) y que la acción carcinógena del dimetilbenzantraceno (DMBA) sobre la mama en ratones *knockout* para el RP depende de la existencia de RP funcionales (Lydon y cols., 1999).

Finalmente, en estudios clínicos de las especies felina, canina y humana se ha observado que el uso de antiprogestágenos tiene efectos favorables sobre las lesiones que expresan RP. La administración de *aglepristona* (un esteroide sintético con una afinidad por el RP 3 veces mayor que la de la progesterona natural), produce la regresión completa

de la *hiperplasia fibroepitelial*, una lesión con RP que se desarrolla bajo el influjo de los progestágenos en la gata (Hayden y cols., 1989; Martín de las Mulas y cols., 2000b; Wehrend y cols., 2001) y de los fibromas vaginales en la perra (Rollón y cols., 2008). Y en la mujer con cáncer de mama avanzado se han observado respuestas favorables (regresión parcial o estabilización de las lesiones) en más de la mitad de las pacientes tras el uso del antiprogestágeno RU486, y en todos los casos, los tumores tenían RP (Maudelonde y cols., 1987; Bakker y cols., 1990; Klijn y cols., 2000).

En la mujer, el cáncer de mama es el tumor más frecuente y se asocia a mortalidad alta, pero existen varios protocolos de tratamiento adyuvante a la cirugía que varían en función de las características de los tumores. El tratamiento endocrino disminuye la frecuencia de recidivas y de metástasis y prolonga la supervivencia de las pacientes (Dickson y Lippman, 1997). El uso de tratamiento endocrino se basa en la detección de receptores hormonales por técnicas IHQ. El 70%, aproximadamente, de los casos expresa RE con o sin RP, y la expresión de receptores es un factor predictivo de respuesta favorable al tratamiento endocrino (Rosai, 1996; Robbins y cols., 1999; Kay y cols., 2011). Por eso, la detección IHQ de receptores hormonales es fundamental para la toma de decisiones terapéuticas.

En la perra con cáncer de mama no se utiliza el tratamiento endocrino aditivo como adyuvante a la cirugía en aquellos casos que

tienen RE y/o RP en las células tumorales. En estudios tanto *in vitro* como *in vivo* se comprobó que el antiestrógeno tamoxifeno tenía efectos antitumorales en la perra pero también que sus efectos agonistas en el aparato reproductor (aumento del tamaño vulvar, infección urinaria, piometra y signos de estro) desaconsejaban su uso clínico en la perra (Else, 1979; Morris y cols., 1993; Sartin y cols., 1993; Hoffmann y Schuler 2000; Sorenmo y cols., 2013). Hasta la fecha, no se han analizado los efectos de los antiprogestágenos sobre los tumores de mama caninos ni *in vivo* ni *in vitro* y los estudios que indican que la progesterona ejerce acciones proliferativas sobre el cáncer de mama a través del RP abren la posibilidad de uso de estos agentes en el tratamiento sistémico del cáncer de mama en la perra.

3. Estudios

3.1. La aglepristona disminuye la proliferación de carcinomas de la mama canina con receptores de progesterona

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Aglepristone Decreases Proliferation in Progesterone Receptor-Positive Canine Mammary Carcinomas

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Background: Progesterone receptor (PR) antagonist aglepristone (RU534) has been used successfully for pregnancy termination and therapy of pyometra, vaginal tumors, and mammary hyperplasia in bitches and queens. All of these conditions share with canine mammary carcinomas the expression of PR.

Objectives: To study the effect of RU534 on proliferation and apoptosis in canine mammary carcinomas in relation to PR expression.

Animals: Twenty-seven nonspayed bitches with mammary carcinomas were treated with either 2 doses of 20 mg/kg RU534 (n = 22, RU534-treated group) or oil placebo (n = 5, control group) on days 1 and 8.

Methods: Tumor samples were collected before (day 1) and after (day 15) treatment for immunohistochemistry. PR expression, proliferation index (PI), and apoptotic index (AI) were determined using antibodies against PR, Ki67, and cleaved lamin A/C antigens, respectively. The effect of treatment on these parameters was analyzed.

Results: Differential expression of PR between day 1 (59.1% PR-positive tumors) and day 15 (36.4% PR-positive tumors) was observed in RU534-treated tumors exclusively. After RU534 treatment, mean PI was significantly decreased in PR-positive but unchanged in PR-negative RU534-treated tumors. A reduction of ≥20% in PI was found in 61.5% of RU534-treated tumors with PR expression. Conversely, no effect on AI was observed after RU534 treatment.

Conclusions and Clinical Importance: Neoadjuvant RU534 treatment had PR expression-related inhibiting effects on proliferation of canine mammary carcinoma cells.

Key words: Apoptosis; Dog; Progesterone receptor; Proliferation.

Surgical excision is the first-line treatment of canine mammary tumors. Approximately one-third of these tumors will recur and metastasize. However, the only type of adjuvant treatment used is chemotherapy.[1] Endocrine therapy using hormone receptor antagonists is well established for the treatment of hormone-dependent human breast cancer because it decreases relapse and metastasis rates and prolongs survival. This therapy is mostly focused on the blockade of estrogen receptors (ER) because they are expressed in approximately 70% of cases.[2] However, progesterone receptor (PR) expression-based therapy currently is under study for selected subsets of patients.[3] Studies in dogs have demonstrated that all benign and two-thirds of malignant mammary tumors express PR.[4–6] This finding raises the possibility of using hormone therapy with PR antagonists in these tumors.

Abbreviations:

AI	apoptotic index
ER	estrogen receptor
H&E	hematoxylin and eosin
PBS	phosphate buffered saline
PI	proliferation index
PR	progesterone receptor
RU534	aglepristone
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

The PR antagonist aglepristone (RU534) has been used successfully in veterinary medicine for pregnancy termination and pyometra treatment as well as to treat proliferative progesterone-dependent diseases such as mammary fibroadenomatous hyperplasia in queens and vaginal tumors in bitches. In both conditions, a tissue reductive effect has been shown.[7–10] The aim of the present study was to evaluate the effect of RU534 on proliferation, apoptosis, and PR expression by immunohistochemical assessment in serial biopsies from primary canine mammary carcinomas collected before and at 15 days after the start of treatment.

Materials and Methods

Animals

A series of 27 female dogs with a histological diagnosis of mammary carcinoma were selected for this study. Additional recruited

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animals with other tumor types were excluded from the present investigation. Recruitment criteria were as follows: (1) dogs were nonspayed; (2) dogs were in any phase of the estrus cycle except for estrus (as determined by vaginal cytology); (3) there were no clinical signs of inflammatory mammary carcinoma; and (4) dogs had 1 measurable mammary nodular lesion (≥5 mm) and had no lung metastases (as determined by 2 thoracic radiographs). All the owners gave informed consent to include their pets in this study.

Treatment Protocol

The RU534-treated group of dogs (n = 22) received 2 subcutaneous injections of 20 mg/kg of RU534[a] on days 1 (1st visit) and 8, whereas control dogs (n = 5) received oil vehicle injections at the same time points. A core biopsy was taken on day 1 before to the first injection of RU534 or oil vehicle. All patients underwent complete surgical excision of the tumor at day 15.

Tissue Samples Fixation and Processing

Both core biopsies and surgical specimens were fixed in 10% buffered formalin for 24–72 hours and embedded in paraffin wax. Small tumors (< 1 cm in diameter) were entirely included, whereas sequential segments 5 mm apart were cut from larger tumors to provide tissue blocks. The area from which the core biopsy had been taken was identified by the surgeon in the surgical specimen with suture material. After dehydration and embedding in paraffin wax, sections (3 μm) were cut from each block and stained with hematoxylin and eosin (H&E).

Histological Examination

The tumors were classified as carcinomas using H&E-stained tissue sections and the diagnostic criteria proposed by the World Health Organization Classification of Tumors in Domestic Animals.[11] Cases with ischemic necrosis in the incision biopsy were excluded from the present investigation.

Immunohistochemical Techniques

The monoclonal mouse anti-human Ki67 antigen (clone MIB-1) isotype IgG_1[b] diluted 1 : 75, the monoclonal mouse PR antibody (clone 10A9) isotype IgG_2[c] diluted 1 : 500, raised against the recombinant hormone-binding domain of human PR (922AGMVKPLLFHKK933, the sequence is 100% homologous to the canine counterpart), and the polyclonal rabbit anti-human cleaved lamin A/C (small subunit) antibody[d] diluted 1 : 100 were used for the detection of proliferative activity, PR expression, and apoptosis, respectively. A commercial diluent[b] was used. Heat-induced antigen retrieval in a water bath at 95–99°C (MIB-1 and PR antibodies) or in a steam pressure cooker[e] at 95°C (lamin A/C antibody) with 0.01 M citrate buffer (pH 6.0) for 40 minutes (MIB-1 antibody), 25 minutes (PR antibody), or 20 minutes (lamin A/C antibody) were used. After cooling for approximately 30 minutes at room temperature, sections were covered with 10% normal goat serum in phosphate buffered saline (PBS) for 30 minutes before incubation with the primary antibodies for 18 hours at 4°C (MIB-1 and PR antibodies) or directly incubated for 1 hours at room temperature (lamin A/C antibody). The avidin-biotin-peroxidase complex (MIB-1 and PR antibodies)[f] or the streptavidin biotin-peroxidase complex (lamin A/C antibody)[b] were applied for 1 hour at room temperature. The chromogen, 3,3-diaminobenzidine tetrahydrochloride[g] diluted 0.035% in 0.05 M Tris containing 0.3% of hydrogen peroxide was applied to the slides for 1 minute at 20–22°C (MIB-1 and PR antibody). The 3-amino-9-ethyl carbazole substrate chromogen[b] was applied for 10 minutes (lamin A/C antibody). For negative control purposes, the primary antibodies were replaced by mouse IgG_1[b] and IgG_2[b] diluted as the primaries (MIB-1 and PR antibodies, respectively) or PBS (lamin A/C antibody). As positive control tissues, canine lymph node, uterus, and formalin-fixed and paraffin wax-embedded ultraviolet-irradiated canine keratinocytes[12] were used for MIB-1, PR, and lamin A/C antibodies, respectively. The normal mammary gland tissue found in the vicinity of the carcinomas served as an internal positive control in every assay.

Scoring Methods

PR Expression. The staining was nuclear and the tumors were classified as positive when labeling was observed in more than 10% of tumor cells counted in 4 representative randomly selected neighboring, nonoverlapping high-power fields (approximately 1,000 tumor cells).[13]

Proliferation Index (PI). To determine the PI, digital pictures of sections labeled with the anti-Ki67 antibody were taken at a 40× magnification from 4 randomly selected neighboring, nonoverlapping fields of each tumor. Labeled tumor cell nuclei were considered positive regardless of the labeling intensity. The number of positive and negative tumor cells was counted with a digital pen tablet.[h] A minimum of 1,000 tumor cells were counted per case. The PI was calculated with the Image-Pro Plus 4.5 software[i] and expressed as the percentage of positive cells related to the total number of cells. The proliferation response of tumors to treatment was assessed in 2 different ways: (1) by comparing mean PI scores at individual time points (day 1, day 15) and (2) by classifying a ≥20% change in PI between day 1 and day 15.[14]

Apoptotic Index (AI). To evaluate apoptosis, cleaved lamin A-positive cells within tumor tissue areas were counted using snapshots of 10 randomly selected fields of each tumor collected at a 40× magnification (Imagescope version 10.2.1.2314).[j] The counts were performed independently by 2 observers and the results were averaged. The AI was expressed as the percentage of labeled cells related to the total number of tumor cells. To assess the effect of treatment on tumor cell apoptosis, results were evaluated in 2 ways analogously as described above for the PI.

Statistical Analysis

Statistical analysis was carried out by the GraphPad Software 3.05.[k] The values were evaluated for approximate normality of distribution by by the Kolmogorov-Smirnov test. Differences between PR expression, PI, and AI of control and RU534-treated tumors before and after treatment were assessed by unpaired *t*-tests. Differences between the means of RU534-treated tumors before and after treatment were assessed by paired *t*-tests. Correlation analyses were performed by Spearman's nonparametric correlation coefficient. A *P*-value < .05 was regarded as statistically significant.

Results

The animals ranged in age from 5 to 16 years of age (mean, 10.6±0.5 years) and were of various pure (n = 14) and mixed (n = 13) breeds. Histological classification of lesions is shown in Table 1. Before treatment (day 1), 60% control and 59.1% RU534-treated tumors expressed PR. A representative PR labeling of tumor cell nuclei is shown in Figure 1. A change in PR expression after treatment was observed in the R534-treated group exclusively (Table 2). Thus, 5 of 13 PR-positive tumors at day 1 became PR-negative at day 15. Conversely, 1 of 9 PR-negative tumors at day 1 was found to be PR-positive at day 15. Altogether, after treatment only 36.4% of RU534-treated tumors expressed PR.

Tumor cell proliferation and apoptosis were assessed using antibodies against Ki67 and cleaved lamin A, respectively. A representative Ki67 labeling of tumor cell

Table 1. Histological type of carcinomas.[11]

Number	Histological Type
14	Complex carcinoma
6	Simple carcinoma
5	Carcinoma in benign tumor
1	Carcinosarcoma
1	Squamous cell carcinoma

nuclei is shown in Figure 2. Cleaved lamin A was detected in the cytoplasm and nucleus of cells with morphology consistent with apoptosis, as well as in the nucleus of some morphologically normal cells as shown in Figure 3. The overall median pretreatment PI was 7.99% (range, 1.5–16.4%) and the overall median pretreatment AI was 1.22% (range, 0.1–4.4%). No association was observed between these 2 variables ($r = 0.2$, $P = .2$). Similarly, no association was observed between PI and AI when tumors were grouped as PR-positive ($r = 0.4$, $P = .1$) and PR-negative ($r = -0.002$, $P = .9$).

PI and PR Expression

A significant decrease in PI after treatment with RU534 was observed in PR-positive tumors of the R534-treated group exclusively. Thus, as indicated in Table 3, the mean PI of PR-positive RU534-treated tumors was 7.7% before and 4.3% after treatment ($P = .03$). Seventy-eight percent of the R534-treated cases with decreased PI after treatment expressed PR. In the control group, only one of the PR-positive cases with PR expression had a reduction in PI at day 15. When a change $\geq$20% of the PI was considered as a threshold, 8 of 13 (61.5%) R534-treated cases with PR expression had a PI reduction, whereas none of the cases with PR expression had a PI reduction in the control group ($P = .0003$). No significant PI increase was observed in the PR-negative R534-treated group after RU534 treatment.

No significant PI decrease was observed in both RU534-treated (7.2% at day 1, 6.9% at day 15) and control (11.0% at day 1, 9.3% at day 15) tumors ($P = .1$ and .3, respectively).

AI and PR Expression

No change in AI was observed either in PR-positive or in PR-negative tumors of the RU534-treated group.

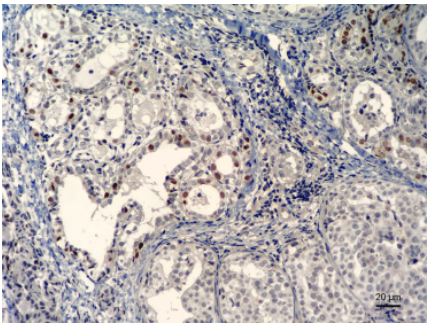

Fig 1. Simple mammary carcinoma. The nuclei of 10% tumor cells are labeled for progesterone receptors (brown color). ABC immunohistochemical method. Bar = 20 µm.

Thus, as indicated as Table 4, the mean AI of PR-positive RU534-treated tumors was 1.5% before and 1.0% after treatment and 1.4% and 1.1 in the control group, respectively (Table 4).

Discussion

The PR antagonist RU534 decreased the PI of canine mammary carcinomas in a PR expression-dependent manner. This finding points to an influence of PR on the growth control of these tumors and raises the possibility of use of PR antagonists in the treatment of tumors of the mammary gland of the bitch.

The clinical benefits of endocrine therapy for women with hormone-sensitive breast cancer (with ER expression, PR expression, or both) are well established. Accordingly, ER and PR are measured routinely in order to select those patients who will enter the endocrine therapy protocol.[2] In veterinary medicine, however, no endocrine therapy, neither adjuvant nor neoadjuvant, currently is used. Former trials using tamoxifen were not successful because of strong estrogenic effects on the uterus.[1] However, the potential benefits of using PR antagonists in dogs with mammary tumors have not been explored despite the fact that PR is more often expressed than ER in the bitch.[4–6] Ours is the first study to examine the effect of neoadjuvant RU534 in canine mammary carcinomas.

Table 2. PR expression in tumors before (day 1) and after treatment (day 15) with RU534 or vehicle (control group).

	Number of Tumors with Indicated PR Status			
	PR+		PR−	
	Day 1	Day 15	Day 1	Day 15
Control group (n = 5)	3	3	2	2
RU534-treated group (n = 22)	13	8	9	14

PR+, progesterone receptor-positive; PR−, progesterone receptor-negative.

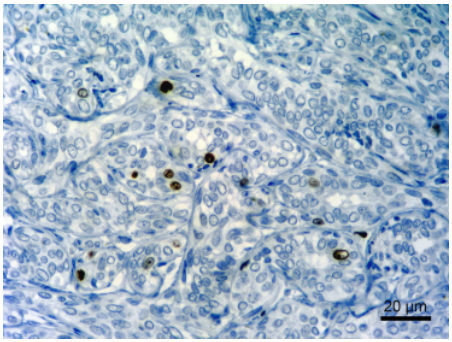

Fig 2. Simple mammary carcinoma. The nuclei of 5% tumor cells are labeled for Ki67 antigen. ABC immunohistochemical method. Bar = 20 µm.

The proliferative response to aglepristone was related to PR status of mammary carcinomas. Thus, a significant decrease of PI on day 15 was observed in treated tumors expressing PR on day 1. Furthermore, the reduction in PI was ≥20% in 61.5% of those cases. These results show that RU534 has an antiproliferative effect in canine mammary carcinomas with PR expression, although sample size is small to make a strong conclusion. Similar findings on PI have been reported after 14 days of neoadjuvant endocrine therapy in human patients.[15] The response after only 2 weeks of treatment suggests that RU534 could be useful for the neoadjuvant treatment of canine mammary tumors.

On the contrary, treatment with the PR antagonist RU534 was not associated with a significant decrease of AI of PR-positive carcinomas. Data from RU534-treated tumors suggest that tumor growth reflects changes in the balance between apoptosis and proliferation. The in vivo relationship of apoptosis to proliferation is less well understood. In our material, we have not found any relationship between PI and AI before treatment. Other authors found a positive relationship between PI and AI of mammary gland tumors of dogs.[16] However, these authors used the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method to detect apoptosis and their case material included a considerable number of benign tumors. We examined apoptosis in canine mammary tumors with a caspase substrate, a method that might be more specific than TUNEL. Lamin A is a nuclear membrane structural component of differentiated eukaryotic cells. This intermediate-sized filament protein is cleaved by the executor caspase-6 and the cleavage and subcellular localization kinetics have been described in cells undergoing apoptosis.[17] In agreement with that study, immunohistochemistry for cleaved lamin A in our case material detected cells at different stages of apoptosis, with staining restricted to the nuclear membrane in putative early stages, and more dispersed nuclear and cytoplasmic staining in cells showing morphological features compatible with more advanced stages of apoptosis. In women with breast cancer, AI has been evaluated after neoadjuvant chemotherapy with inconsistent results.[14,18] Several factors may account for these discrepancies, including the type of marker used, the type of tumors examined, the time point of tissue collection during treatment, and others.

A change in PR expression after treatment with RU534 was observed in the RU534-treated group but not in the control group. Thus, 38.5% of PR-positive tumors treated

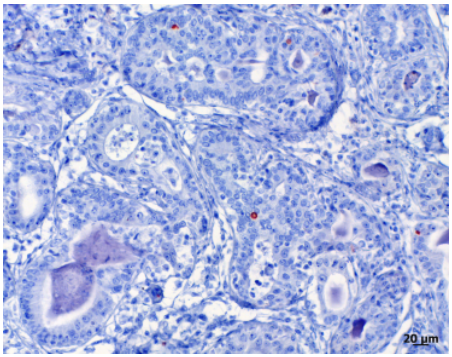

Fig 3. Complex mammary carcinoma. Single cells (1%) within the tumor are labeled for cleaved Lamin A. LSAB immunohistochemical method. Bar = 20 µm.

Table 3. Proliferation index (PI) before (day 1) and after treatment (day 15) with RU534 or vehicle (control group) in tumors grouped according to progesterone receptor status at day 1.

	Mean ± SD of PI (%)		
	PR Status at Day 1	Day 1	Day 15
Control group	PR+ (n = 3)	8.1 ± 5.3	8.4 ± 2.4
	PR− (n = 2)	15.3 ± 1.5	10.7 ± 2.0
RU534-treated group	PR+ (n = 13)	7.7 ± 3.9	4.3 ± 4.5[a]
	PR− (n = 9)	6.6 ± 4.7	10.5 ± 9.0

PR+, progesterone receptor-positive; PR−, progesterone receptor-negative; SD, standard deviation.
[a] $P < .05$ versus day 1. Paired t test.

Table 4. Apoptotic index (AI) before (day 1) and after treatment (day 15) with RU534 or vehicle in tumors grouped according to progesterone receptor status at day 1.

	Mean ± SD of AI (%)		
	PR Status at Day 1	Day 1	Day 15
Control group	PR+ (n = 3)	2.1 ± 1.9	2.3 ± 1.2
	PR− (n = 2)	0.4 ± 0.3	0.6 ± 0.2
RU534-treated group	PR+ (n = 13)	1.5 ± 1.3	1.0 ± 0.5
	PR− (n = 9)	1.4 ± 0.8	1.1 ± 0.9

PR+, progesterone receptor-positive; PR−, progesterone receptor-negative; SD, standard deviation.
$P < .05$ versus day 1. Paired t test.

with RU534 became negative. These data suggest that treatment with RU534 may be associated with down-regulation of PR, a finding already described in mammary tumors of rats after treatment with the aglepristone analog RU486.[19] The loss of PR may be associated with its phosphorylation after ligand binding. RU486 has been shown to phosphorylate the PR without transcriptional effects. However, phosphorylated PR is targeted for degradation.[20] These cells with degraded PR may have been induced to differentiate terminally.[21] The decrease of PI after RU534 treatment of dogs bearing mammary carcinomas is compatible with the selective preservation of clones of slowly proliferating cells during treatment, as previously suggested by others studying human tumors.[14]

The approach of using core biopsies to assess molecular markers before and after treatment as we have used in the present study has become widely used in a number of studies in human medicine. It has, however, some limitations, including the effects of intratumoral heterogeneity in the evaluation of the response to treatment. We have tried to avoid this limitation by identifying the area where the core biopsy had been taken with suture material in the surgical specimen. In this way, tissue samples used for comparison studies were taken from the same area of the lesion. In addition, incision biopsies with necrotic areas were not used in this study. Also, the influence of overall tumor size heterogeneity on these results should be evaluated further.

In conclusion, immunohistochemical staining for Ki67, cleaved lamin A, and PR antigen has provided objective measures of the major biological effects of therapy with RU534 in canine mammary carcinoma, but further studies with both more samples and correlation data between these biological effects and clinical variables should be carried out. The present data indicate that selection for neoadjuvant endocrine therapy should be based primarily on hormone receptors status, because dogs treated with neoadjuvant endocrine therapy are most likely to respond and derive clinical benefit when their tumors are a PR-positive.

Footnotes

[a] Alizine, Virbac, France
[b] Dako, Barcelona, Spain
[c] Immunotech, Marseille, France
[d] Cell Signaling Technology, Danvers, MA
[e] Pascal, Dako
[f] Vector Laboratories; Burlingame, CA
[g] Sigma, Saint Louis, MO
[h] Volito 2, Wacom Europe GmbH, Krefeld, Germany
[i] Media Cybernetics, Silver Spring, MD
[j] Perio, Vista, CA
[k] GraphPad Software Inc, San Diego, CA

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3.2. Estudio de las isoformas del RP mediante RT-qPCR en displasias y tumores de mama caninos fijados en formol e incluidos en parafina

Veterinary Pathology

Progesterone receptor isoforms analysis by RT-qPCR in formalin-fixed paraffin-embedded canine mammary dysplasias and tumors.

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Keywords:	Dog < Domestic Mammals < Species, Mammary tumors, progesterone receptor, isoform A, isoform B, RT-qPCR
Abstract:	In the dog, cloning and sequencing of the progesterone receptor gene has shown two isoforms, A and B, transcribed from a single gene. Distribution of isoforms A and B has been studied in canine mammary lesions by Western blot analysis exclusively. The aim of this study was to analyze progesterone receptor and its isoforms in formalin-fixed, paraffin-embedded tissue samples in canine mammary lesions (4 dysplasias, 10 benign tumors and 46 carcinomas) by one-step SYBR Green RT-qPCR. Progesterone receptor was expressed in 75% dysplasias, all benign tumors and 59% carcinomas. Carcinomas and simple type carcinomas had the lowest expression levels. A high concordance was found between RT-qPCR and immunohistochemistry labeling. Isoforms A and B were successfully amplified with correlation coefficients of 0.99 and efficiencies close to 2 and expressed in all types of lesions analyzed. A predominance of A over B expression was observed in carcinomas and complex adenomas. Tumors with low grade of malignancy had higher mRNA levels of progesterone receptor but no differences were observed in the expression of isoform A and B. Results showed the feasibility of analysis of progesterone receptor at the mRNA level using fixed tissue samples and a highly sensitive RT-qPCR method. They also showed the distribution of progesterone receptor

	isoforms mRNA A and B in dysplasias, benign tumors and malignant tumors of the canine mammary gland for the first time in literature. These findings will allow studying the role of progesterone receptor isoforms in the progression of canine mammary tumors.

Title page

Progesterone receptor isoforms analysis by RT-qPCR in formalin-fixed paraffin-embedded canine mammary dysplasias and tumors.

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Abstract

In the dog, cloning and sequencing of the progesterone receptor gene has shown two isoforms, A and B, transcribed from a single gene. Distribution of isoforms A and B has been studied in canine mammary lesions by Western blot analysis exclusively. The aim of this study was to analyze progesterone receptor and its isoforms in formalin-fixed, paraffin-embedded tissue samples in canine mammary lesions (4 dysplasias, 10 benign tumors and 46 carcinomas) by one-step SYBR Green RT-qPCR. Progesterone receptor was expressed in 75% dysplasias, all benign tumors and 59% carcinomas. Carcinomas and simple type carcinomas had the lowest expression levels. A high concordance was found between RT-qPCR and immunohistochemistry labeling. Isoforms A and B were successfully amplified with correlation coefficients of 0.99 and efficiencies close to 2 and expressed in all types of lesions analyzed. A predominance of A over B expression was observed in carcinomas and complex adenomas. Tumors with low grade of malignancy had higher mRNA levels of progesterone receptor but no differences were observed in the expression of isoform A and B. Results showed the feasibility of analysis of progesterone receptor at the mRNA level using fixed tissue samples and a highly sensitive RT-qPCR method. They also showed the distribution of progesterone receptor isoforms mRNA A and B in dysplasias, benign tumors and malignant tumors of the canine mammary gland for the first time in literature. These findings will allow studying the role of progesterone receptor isoforms in the progression of canine mammary tumors.

Keywords

Dog; mammary tumors; progesterone receptor; isoform A; isoform B; RT-qPCR; immunohistochemistry.

Epidemiologic, clinical and experimental data indicate that canine mammary tumors (CMT) are hormone-dependent, that is, are strongly influenced by ovarian hormones, mainly estrogens and progesterone (P).[35] P mediates its effects through binding to its cognate receptor, the P receptor (PR), which is a member of the nuclear steroid receptor family.[12] PR expression is currently measured by immunohistochemical (IHC) methods which have shown that all benign and two-thirds of malignant CMTs express PR.[7,13,25] Further, the IHC expression of PR is a favorable prognostic indicator[25,34] and a predictive marker of positive response to neoadjuvant administration of antiprogestins[14] in canine mammary carcinoma.

The PR exists as two isoforms, PRA and PRB, which are expressed from a single gene in both humans and rodents.[19] PRA and PRB have been shown to have different functions as well as differential expression levels in breast cancer. Thus, human PRB has been found to be a stronger transcriptional activator than human PRA, due in part to a third activation domain (AF-3) within the N terminal 164 amino acids.[34] On the other hand, human PRA has been shown to act as a repressor which can inhibit other receptors including estrogen receptor and human PRB.[37] PRA and PRB are generally expressed at similar levels in normal mammary tissue, but their ratio is altered with a predominance of PRA and loss of PRB in breast tumors.[6,19,28]

In the dog, cloning and sequencing of the PR gene has shown that the PR has two isoforms, PRA and PRB, transcribed from a single gene.[23] Western blot analysis revealed predominant staining for PRA with less intense staining of PRB in normal, hyperplasic and neoplastic canine mammary tissue samples.[11] Moreover, PRA expression was higher in carcinomas than in normal and hyperplastic tissue.[11]

Nowadays, quantitative real-time PCR (RT-qPCR) is considered to be the most sensitive method for PR isoforms detection since Western blot and IHC methods have

limitations regarding sensitivity of detection.[19,27] Furthermore, there is a growing interest to use the vast archives of formalin-fixed, paraffin embedded (FFPE) tissue samples available in veterinary pathology laboratories for retrospective studies using RT-qPCR.

The aim of this study was to investigate the expression of total PR, PRA and PRB in FFPE canine dysplasias, benign and malignant tumors of the mammary gland of the dog by using a RT-qPCR method. Total expression of PR was also analyzed by IHC for comparison.

Materials and methods

Samples

FFPE tissue samples of 61 lesions of the canine mammary gland were retrieved from the archives of the Department of Comparative Pathology of the University of Córdoba (Spain). These samples were from a former prospective study and thus had a controlled fixation time for 24h to 48h in 10% buffered formalin and were stored as paraffin blocks at 4°C between 0.5-2 years before use. Histological classification[26] and histological grade[21] of lesions are presented in Table 1. Tissue samples from two fresh canine mammary tumors from the clinic were used as controls for the validation of the RT-qPCR and then fixed for 24h in 10% buffered formalin and routinely embedded in paraffin wax.

Extraction, quantification and quality assessment of mRNA

For isolation of RNA from FFPE and fresh samples, the RNase FFPE kit and the RNeasy Protect Mini kit (Qiagen, Copenhagen, Denmark) were respectively used according to manufacturers´ recommendations, and stored at -80 ºC until using.

RNA yields were determined by the spectrophotometric absorbance at 260 nm (A_{260}) measured using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A ratio of absorbance at 260 nm and 280 nm 1.8-2.0 was accepted as "pure". The integrity of total RNA was checked by denaturing agarose gel electrophoresis and ethidium bromide staining showing the respective mRNAs as sharp bands.

RT-PCR assay

Primer design

The canine PR genomic sequence was obtained from NCBI GenBank database under the gene ID NM_001003074. Two primer pairs were designed specifically to target the coding regions of both canine isoforms A and B previously described by Lantinga-van Leeuwen et al. (2000) using Primer3Plus. Moreover, two canine housekeeping genes, the hypoxanthine phosphoribosyl-trasnferase 1 (HPRT1: NM_001003357.1) and canine ribosomal protein L32 (RPL32: NM_001252169.1) were selected for the study and two primer pairs were designed with the same tool. These two genes have shown to be suitable reference genes as internal controls for RT-qPCR in CMTs.[10,18]

Table 2 summarizes the sequences of the forward and reverse primers for PR isoforms and housekeeping genes. Primers spanning or flanking one intron were chosen to minimize inaccuracies due to genomic DNA contamination when it was possible. The specificity of the different primers was checked by performing a BLAST® search showing 100% homology to target genes.

RT-qPCR amplification

RNA was amplified using the LightCycler® 480 Real-Time PCR System. One-step RT-qPCR was performed using the QuantiFast® SYBR® Green RT-PCR (Qiagen,

Denmark) for a total of volume of 10 µl and a template concentration of 5ng/µl according to manufacturer´s recommendations. Thermal cycling conditions were 50 ºC for 10 min (RT step) and 95 ºC for 5 min, followed by 40 cycles of 95 ºC of 10 s and 60 ºC for 30 s. A melting curve analysis was performed following every run to ensure a single amplified product for every reaction (1 cycle of 95 ºC, with continuous acquisition mode and rampe rate of 0.1 ºC/s). RT-qPCR products were analyzed by agarose gel electrophoresis (Fig. 1). Reverse transcription negative controls and non template controls were included. All reactions were performed by triplicate in a 96-well reaction plates (Applied Biosystems, Foster City, CA, USA).

Standard curves generated from series of dilutions (1ng/µl to 20ng/µl) of a known sample to encode the canine PR gene were used to determine qPCR efficiency ($E = 10^{(-1/slope)}$) and to establish the linear range of the assay.

The reliability of the RT-qPCR was defined by calculating the coefficient of variation (CV) of replicates for each analyzed sample (intra-assay variability) and for each plate (inter-assay variability). CV was calculated with the following formula: (SD/Ct average) x 100. Relative mRNA expression was defined as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{TARGET} - Ct_{RPL32/HPTR1}$, and $Ct_{RPL32/HPTR1}$ is the average of the Ct values of the two housekeeping genes for each sample. The amount of PRA was calculated by subtracting the relative amount of PRB from that of total PR.[15,36] The PR positive-status cut-off was established at 0.04 values since it represented the best concordance with IHC results (see below).

IHC assay

For the detection of total PR by IHC, the commercial mouse monoclonal anti-human PR antibody (clone 10A9, Immunotech, Marseille, France) diluted 1:500 and the avidin-biotin-peroxidase complex technique (Vector, Burlingame, CA, USA) were used

as previously described.[7] This antibody is raised against the recombinant hormone-binding domain of human PR located on the C-terminal domain which is a common region of PRA and PRB.[20] For the quantitative analysis of total PR expression, digital pictures were taken at a 40x magnification from 15 randomly selected neighboring, non overlapping fields of each sample labeled with the anti-PR antibody. The number of positive and negative cells was counted with ImageJ software (ImageJ 1.43, National Institute of Health, Maryland, USA). A minimum of 1000 tumor cells was counted per case. PR expression was expressed as the percentage of positive cells related to the total number of cells. The cut-off for the determination of PR positive-status was established in 10%.[14]

Statistical analysis

For statistical data evaluation the software GraphPad Prism 5 version 5.01 (GraphPad Software Inc, San Diego, CA, USA) was used. The values were evaluated for normality analysis using the D´Agostino-Pearson normality test. Mann-Whitney U test was used to analyze RT-qPCR (total PR, PRA and PRB) and IHC (total PR) results with respect to histological types and subtypes and histological grade. Concordance degree between RT-qPCR and IHC methods was estimated by Cohen´s κ coefficient. A P value < 0.05 was regarded as statistically significant.

Results

RT-qPCR validation

A total of 61 FFPE CMTs were processed. The average values of RNA quantity was 46 ng/µl (range 9.7- 189.3 ng/µl) and average purity obtained at 260/280 nm was 1.85 (1.6-2.1). Only one sample (<2%) histologically classified as benign mixed tumor

was excluded due to the low quantity and quality. For canine fresh tissues and their equivalent FFPE tissues the average values of RNA quantity and purity were 242 ng/µl and 1.8 and 180.7 ng/µl and 1.7, respectively.

All RT-qPCR amplification plots displayed adequate amplification curves with an exponential phase followed by a non-exponential phase, ending with a plateau. The melting analysis showed curves with a single peak and adequate melting temperatures (Tm) for housekeeping genes and PR. However, for PRB, the melting curve analysis was characterized by a sharp peak at 91 ºC but multiple extra peaks could be observed at lower Tm, which is consistent with the data from the agarose gels (Fig. 1). Non template controls and reverse transcription negative controls did not show any amplification.

RNA isolated from the two samples of fresh CMTs produced Ct values which were 4 to 6 cycles lower than the Ct values produced from the same RNA isolated from their equivalent FFPE tissues (Table 3). However, in both cases (fresh and FFPE tissues) a clear peak in the melting curve, with the same Tm indicated the purity and specificity of the amplified PCR fragments (Table 3).

Initially, the molecular weight of the PCR-products arising from the primer pairs in Table 2 was verified on agarose gels. The results shown in Table 2 and Fig. 1 verified that the designed primer pairs were specific. A correlation coefficient (R^2) between 0.98 and 0.99 for all qPCR assays and E values between 2.19 and 2.89 were observed. Primer pairs total PR (1), PRB (2), HPTR1 (2) and RPL32 (1) (Table 2) were selected for the experiment based on agarose gel analysis and better R^2 and E values.

The intra-assay variability was lower than 5% in all cases and most of the samples presented CV values from 0.03 to 2.64. The inter-assay variability showed CV values ranging from 0.78 to 1.2%.

Total PR, PRA and PRB analysis by RT-qPCR

Seventy five percent of dysplasias, 100% of benign tumors and 59% of carcinomas were considered positive for mRNA expression of total PR by RT-qPCR (Table 4). Benign tumors and dysplasias presented equal RT-qPCR values (0.07±0.04 and 0.07±0.06, respectively) (Fig. 2). Expression of PR was lowest in carcinomas (0.05±0.008) (P= 0.02) with simple epithelial-type carcinomas showing the lowest values (0.03±0.02) (Fig. 2). Carcinomas grades 1 and 2 showed significantly higher expression of total PR than carcinomas grade 3 (P = 0.02) (Fig. 3).

While PRA and PRB expression were similar in dysplasias and benign tumors, PRA expression in carcinomas was higher than PRB expression (0.03 and 0.01, respectively) (P = 0.0006) (Fig. 4). Differences between PRA and PRB were higher in complex carcinomas (P < 0.0001) and simple carcinomas (P = 0.07) than in carcinomas in benign tumors (Fig. 4). When the histological subtypes of benign tumors were analyzed separately, complex adenomas but not benign mixed tumors had higher expression of PRA with respect to PRB (P = 0.01) (Fig. 4). No differences in PRA and PRB expression were found with respect to the histological grade of carcinomas.

PR expression by IHC

All dysplasias, 90% of benign and 54% of malignant tumors presented PR labeling in the nuclei of luminal-type epithelial cells (Fig. 5). In addition, the cytoplasm of myoepithelial cells of complex and mixed tumors was also labeled (Fig. 6). Among carcinomas, simple carcinomas showed the lowest expression of PR. Carcinomas with histological grades 1 and 2 had higher PR labeling than carcinomas with histological grade 3 (P = 0.045) (Fig. 3).

Concordance between IHC and RT-PCR

The percentage of lesions considered PR-positive with RT-qPCR (66.7%) and IHC (65%) was similar. A concordance was found with an overall agreement of 75% between both methods (Kappa index 0.4). Thus, 7 cases were PR negative on RT-qPCR but PR positive on IHC while 8 cases were PR positive by RT-qPCR and PR-negative by IHC (Fig. 7). According to the histological type, the highest concordance between methods was found in lesions classified as benign tumors (90%).

Discussion

In the present study, the analysis of total PR, PRA and PRB mRNA expression in canine mammary lesions is presented for the first time in literature by using a RT-qPCR method in formalin-fixed, paraffin embedded tissue samples. Seventy five percent of dysplasias, 100% of benign tumors and 59% of carcinomas were considered positive for mRNA expression of total PR by RT-qPCR. These figures were similar to those obtained with the gold standard IHC method, which served as control. PRA and PRB mRNA expression were found in all types of lesions analyzed and a predominance of PRA over PRB expression was observed in carcinomas and complex adenomas.

Both IHC and Western Blot methods have been used to analyze PRA and PRB isoforms in human tissue samples although some authors point to problems related to the specificity of some commercially available antibodies to distinguish between them.[19,28,32] In the dog, gene expression of the PR has been previously reported using fresh or frozen tissue samples,[5,11,23] but no data are available about gene expression of PR isoforms in FFPE canine tissues by RT-qPCR, a method considered to be the most sensible method for the detection of PRA and PRB.[19] The nucleotide sequence of canine PR gene and the specific sequences for PRA and PRB were identified from fresh mammary tissues.[23] Both isoforms contain the hormone binding domain, a highly

conserved region, and a DNA binding domain but the former lacks a less conserved amino-terminal sequence which is unique for the longer PRB isoform.[23] In the present study, total PR, PRA and PRB mRNA were amplified by using canine-specific primers from archival FFPE samples with satisfactory results. Fresh and FFPE samples showed identical Tm values and agarose gel bands, a finding that supports the reliability of the FFPE data. The use of RT-qPCR for gene amplification from FFPE tissue samples may be affected by the process of fixation and embedding which must be taken into account in order to avoid a negative impact on the quality and usefulness of extracted RNA.[1,32] Therefore, we employed samples which had been fixed for less than 48 h and stored no longer than 2 years at 4 ºC. In these conditions, all samples examined were considered suitable for the analysis showing acceptable purity ratio, yield and integrity of RNA. Another critical factor to take into account for RT-qPCR is primers design.[19] Consequently, canine specific primer pairs were designed to produce an amplicon smaller than 100 bp to ensure that the sequences were unique for the template since it has been shown to improve RT-qPCR efficiency.[29] Investigated transcripts showed acceptable E rates in the range from 1 to 20 ng RNA input with high linearity. To compensate the inter-assay variation, a standard curve for a calibrator was run in each experiment under the same conditions as used for the examined samples. The inter- and intra-assay variability was very low; and the CV values remained below 3% for both isoforms in most of the cases. A CV up to 5% is considered acceptable and has no negative impact on the interpretation of results.[9] Accordingly, the RT-qPCR assay standardized in the present study was highly reliable and robust in terms of sensitivity, repeatability and reproducibility for the detection of total PR, PRA and PRB.

Seventy five percent of dysplasias, 100% of benign tumors and 59% of carcinomas were considered positive for mRNA expression of total PR by RT-qPCR.

These figures were similar to those obtained with the gold standard IHC method, which served as control. Thus, all dysplasias, 90% of benign and 54% of malignant tumors presented PR labeling in the nuclei of luminal-type epithelial cells.[25]. Nuclear labeling is considered to be the specific labeling for PR in FFPE canine mammary tissue samples although cytoplasmic staining of myoepithelial cells has been described.[23,25]. PR expression was lower in carcinomas compared to benign tumors and dysplasias both by RT-qPCR and IHC. PR expression has been shown to be lower in carcinomas than benign tumors and dysplasias when using IHC methods.[25,35] Present findings also confirm former data concerning the lower expression level of PR in simple epithelial-type carcinomas than complex or mixed subtypes and the direct relationship between PR expression and lower histological grades of malignancy.[25] A high concordance was found between PR expression by RT-qPCR and IHC, as described in human studies.[3,17,30] There were 8 cases PR-positive by RT-qPCR which were classified as PR-negative by IHC. The IHC expression of PR in the cytoplasm of myoepithelial cells could have contributed to "false" positive results in RT-qPCR as the presence of cytoplasmic PR staining in myoepithelial cells is in line with the expression of PRB in these cells.[8,11] On the other hand, the loss of tissue in the paraffin block during the development of the technique might be the responsible for the discrepancies in those cases where IHC detected the PR antigen but RT-qPCR did not amplify the mRNA.

Analysis of PRA and PRB mRNA showed that both PR isoforms were found in all types of lesions analyzed. Further, PRA and PRB were expressed at similar levels in canine dysplasias and benign mixed tumors but carcinomas and complex adenomas had higher PRA than PRB expression levels. These findings are similar to those obtained by Western blotting in the canine mammary tissues.[11] In normal human breast, PRA and PRB are generally expressed at similar levels but at least in some breast cancers, their

ratio is dysregulated with a predominance of PRA over PRB.[4,16,22] It is thought that the coordinate expression of both isoforms is required for the normal P response of the mammary gland and that deregulation of this ratio appears early in tumorigenesis.[28] The dissimilar expression of PR isoforms might be of importance for the response of the tumor to endocrine treatment since a high ratio of PRA/PRB has been associated with poorer outcome in patients undergoing hormonal therapy.[16] However, a predominance of isoform A has also been described in some benign breast lesions such as atypical ductal hyperplasias.[28] In humans and rodents, PRA and PRB are expressed in the luminal epithelium.[2,28] In the rat, however, PRA is expressed in the luminal epithelium whereas PRB is expressed in both luminal and myoepithelial cells.[19] Moreover, PRA has been reported to be present in nuclei whereas PRB is found in the cytoplasm.[8,24] The cytoplasmic staining of myoepithelial cells found in this study may be related with PRB expression while the higher expression of PRA mRNA in complex tumors could be attributed to the incomplete or aberrant immunophenotype described in neoplastic myoepithelial cells.[31] Also, differences between human and canine distribution of PRA and PRB in mammary lesions may exist.

Finally, while expression of PR was significantly higher in well-differentiated carcinomas (grade 1 and 2) with both RT-qPCR and IHC methods,[25,35] no differences in PR isoforms distribution were found with respect to histological grade of carcinomas. In human breast cancer, a predominance of isoform A has been associated with higher histological grades[4] in studies based on protein (IHC and Western blot) and not mRNA expression of PR isoforms.

The present study demonstrated the feasibility of analysis of PR at the mRNA level using formalin fixed, paraffin embedded tissue samples and a highly sensitive RT-qPCR method. It has also shown the distribution of PR isoforms A and B mRNA in

dysplasias, benign tumors and malignant tumors of the canine mammary gland for the first time in literature. In addition, a predominance of PRA over PRB seems to be present in CMTs which may be critical in the prognosis and therapeutic decision of these tumors. These findings will allow studying the role of PR isoforms in the progression of CMTs.

Conflict of interest statement

None of the authors of the above manuscript has declared any conflict of interest.

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Tables

Table 1: Histological classification of lesions and histological grade of malignant tumors used in the study.

	n
Histological Classification of lesions	
Total	61
Dysplasias	4
Benign tumors	10
Complex adenomas	6
Benign mixed tumors	5
Carcinomas	46
Complex carcinomas	19
Simple carcinomas	16
Carcinoma in benign tumors	11
Histological grade of carcinomas	**n (%)**
Total	46
Grade 1	22 (48)
Grade 2	17 (37)
Grade 3	7 (15)

Table 2

Primers sequences and product length for RT-qPCR amplification.

	Primer Forward	Primer Reverse	Product Length
PR(1)[a]	5'-GGCTTGCCGCAGGTGTACCA-3'	5'- ACTGTGGGCTCTGGCTGGCA-3'	**73 bp**
PR(2)	5'- GGTCCTTGGAGGTCGAAAAT-3'	5'-ACAGGTTGTGGGAGAGCAAC-3'	**84 bp**
PRB(1)[b]	5'-GCGGACGGGAAAGGATGCCC-3'	5'-GACGTCGGGACTCGGGCTCT-3'	**86 bp**
PRB(2)	5´-CCCGGGCGGATCCGAGACT-3'	5´-ACTGTGGGCTCTGGCTGGCA-3	**86 bp**
HPTR1(1)[c]	5'-ATGGACAGGACTGAGCGGCTT-3'	5'-CCTTGAGCACACAGAGGGCTACG -3'	**82 bp**
HPTR1(2)	5'-TGCAGACTTTGCTTTCCTTGGTCA-3'	5'-TCGAGGGGTCCTTTTCACCAGCA -3'	**81 bp**
RPL32(1)[d]	5'-GGCTGCCCTCAGACCTCTGGT -3'	5'-TCGGTCTGACTGGTGCCGGA -3'	**79 bp**
RPL32(2)	5-GGACCAAGAAGTTCATCCGGCACC-3'	3'-TGCCTCTGGGTTTCCGCCAG-5'	**75 bp**

[a] Total progesterone receptor; [b] Progesterone receptor isoform B; [c] Hypoxanthine phosphoribsyl-transferase 1; [d] Canine ribosomal protein L32.

Table 3

Ct values and melting temperatures (Tm) from fresh and FFPE samples for the two housekeeping genes (RPL32 and HPTR1), PR and PRB.

	RPL32		HPTR1		PR		PRB	
	Fresh (Ct;Tm)	FFPE (Ct;Tm)	Fresh (Ct;Tm)	FFPE (Ct;Tm)	Fresh (Ct;Tm)	FFPE (Ct;Tm)	Fresh (Ct;Tm)	FFPE (Ct;Tm)
Sample 1	15.9; 81 ºC	22.3; 81ºC	22; 81 ºC	28.5; 81 ºC	23.9; 83 ºC	30.6; 83 ºC	30.7; 91 ºC	35; 91 ºC
Sample 2	16.2; 81 ºC	22.8; 81ºC	22.6; 81ºC	28.5; 81 ºC	27; 83 ºC	31; 83 ºC	31; 91 ºC	34.5; 91 ºC

[a] Canine ribosomal protein L32; [b] Hypoxanthine phosphoribsyl-transferase 1; [c] Total progesterone receptor; [d] Progesterone receptor isoform B.

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Table 4: PR-positive cases of dysplasias, benign and malignant tumors by RT-qPCR and IHC.

Histological Classification	RT-qPCR, n (%)	IHC[a], n (%)
Dysplasias	3 (75)	4 (100)
Benign tumors	10 (100)	9 (90)
Complex adenomas	6 (100)	5 (83)
Benign mixed tumors	4 (100)	4 (100)
Carcinomas	27 (59)	25 (54)
Complex carcinomas	15 (79)	14 (74)
Simple carcinomas	8 (50)	6 (37.5)
Carcinoma in benign tumors	4 (36)	5 (45.5)
Total	40 (66.7)	39 (65)

[a] **Immunohistochemistry**

Figure legends

Fig. 1. Representative ethidium bromide-stained gel electrophoresis of canine housekeeping genes from a FFPE canine mammary tumor. The PCR products were obtained after RT-qPCR on the LC480 system. The product size and primer pairs (see Table 1) are indicated in the figure. NTC: Non template control. RT control: Reverse transcription negative control.

Fig. 2. Expression of total PR in the different histological types of tumors and histological subtypes of carcinomas by RT-qPCR. Bars with asterisk differ at ($P < 0.05$).

Fig. 3. Expression of total PR by RT-qPCR (left Y axis) and by IHC (right Y axis) according to the histological grade of tumor. Black columns represents low-grade (grade 1 and 2) tumors and white columns high-grade (grade 3) tumors. Bars with asterisk differ at ($P < 0.05$).

Fig. 4. Expression of PRA (black column) and PRB (white column) in dysplasias, benign tumors and carcinomas and histological subtypes of carcinomas and benign tumors by RT-qPCR. Bars with asterisk differ at ($P < 0.05$).

Fig. 5. Immunohistochemical PR labeling is seen in the nuclei of tumor epithelial cells. 40x magnification. ABC method.

Fig. 6. Immunohistochemical PR labeling is seen in both the nuclei of tumor epithelial cells and the cytoplasm of tumor myoepithelial cells. 40x magnification. ABC method.

Fig. 7. Comparison of RT-qPCR values versus IHC assessment. The Yaxis shows PR expression values by RT-qPCR and X axis shows PR-positive and PR-negative samples according IHC. The cut-off for PR-positive status by RT-qPCR is indicated by the horizontal red line. Black points show discrepancy between both methods.

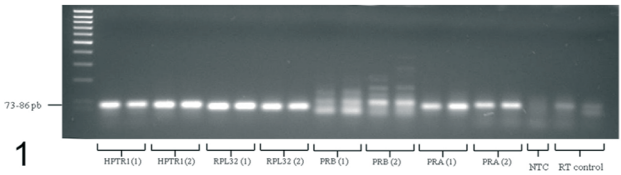

Fig. 1. Representative ethidium bromide-stained gel electrophoresis of canine housekeeping genes from a FFPE canine mammary tumor. The PCR products were obtained after RT-qPCR on the LC480 system. The product size and primer pairs (see Table 1) are indicated in the figure. NTC: Non template control. RT control: Reverse transcription negative control.
90x24mm (300 x 300 DPI)

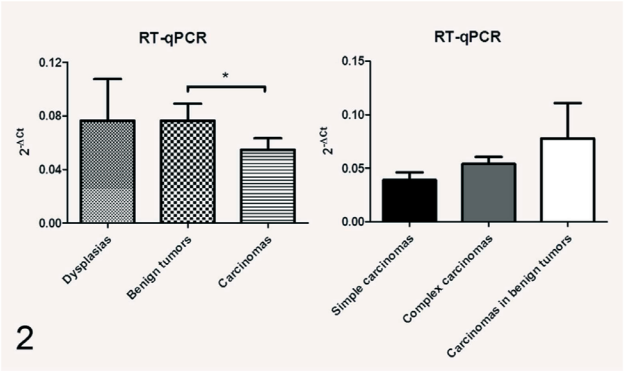

Fig. 2. Expression of total PR in the different histological types of tumors and histological subtypes of carcinomas by RT-qPCR. Bars with asterisk differ at ($P < 0.05$).
90x53mm (300 x 300 DPI)

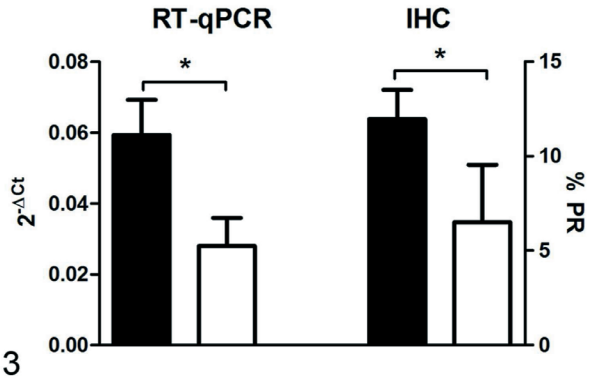

Fig. 3. Expression of total PR by RT-qPCR (left Y axis) and by IHC (right Y axis) according to the histological grade of tumor. Black columns represents low-grade (grade 1 and 2) tumors and white columns high-grade (grade 3) tumors. Bars with asterisk differ at ($P < 0.05$).
90x59mm (300 x 300 DPI)

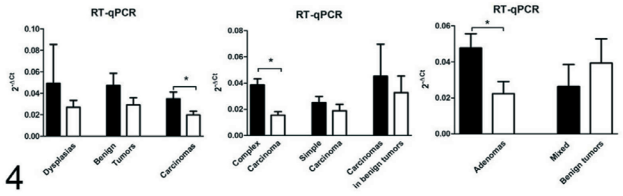

Fig. 4. Expression of PRA (black column) and PRB (white column) in dysplasias, benign tumors and carcinomas and histological subtypes of carcinomas and benign tumors by RT-qPCR. Bars with asterisk differ at ($P < 0.05$).
90x29mm (300 x 300 DPI)

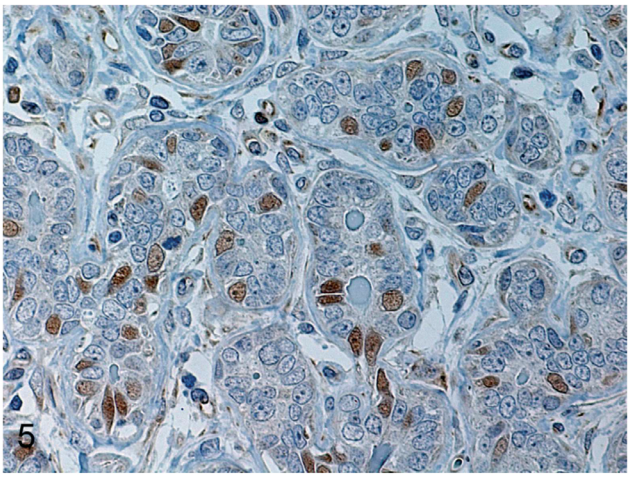

Fig. 5. Immunohistochemical PR labeling is seen in the nuclei of tumor epithelial cells. 40x magnification. ABC method.
90x67mm (300 x 300 DPI)

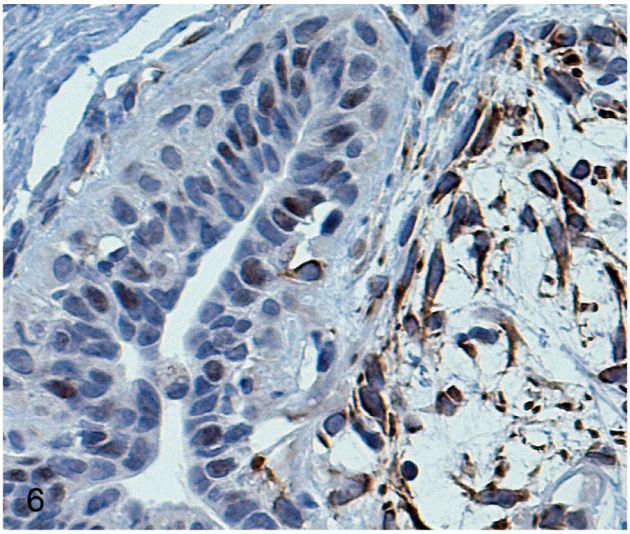

Fig. 6. Immunohistochemical PR labeling is seen in both the nuclei of tumor epithelial cells and the cytoplasm of tumor myoepithelial cells. 40x magnification. ABC method.
90x75mm (300 x 300 DPI)

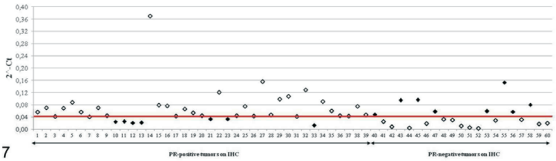

Fig. 7. Comparison of RT-qPCR values versus IHC assessment. The Yaxis shows PR expression values by RT-qPCR and X axis shows PR-positive and PR-negative samples according IHC. The cut-off for PR-positive status by RT-qPCR is indicated by the horizontal red line. Black points show discrepancy between both methods.
180x53mm (300 x 300 DPI)

Decision Letter (VET-13-FLM-0173)

From: a.groene@uu.nl

To: v22gulus@uco.es

CC:

Subject: Veterinary Pathology - Decision on Manuscript ID VET-13-FLM-0173

Body: 09-Aug-2013

Dear Ms. Guil-Luna:

Manuscript ID VET-13-FLM-0173 entitled "Progesterone receptor isoforms analysis by RT-qPCR in formalin-fixed paraffin-embedded canine mammary dysplasias and tumors." which you submitted to Veterinary Pathology, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

The reviewer(s) have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript. Please be sure to include a rebuttal letter with the following:
1- Number and list the Editor's and each reviewers' point
2- Address each point indicating how the manuscript has been revised

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Once again, thank you for submitting your manuscript to Veterinary Pathology and I look forward to receiving your revision.

Sincerely,

Dr. Matti Kiupel
Associate Editor, Oncology

Dr. Andrea Gröne
Editor, ECVP
Veterinary Pathology
a.groene@uu.nl

Reviewer(s)' Comments to Author:

Reviewer: 1
Comments to the Author
The aim of this study was to investigate the expression of total PR, PRA and PRB in FFPE canine dysplasias, benign and malignant tumors of the mammary gland of the dog by using a RT-qPCR method. Total expression of PR was also analyzed by IHC for comparison.

The proposal is interesting however some important points need to be better discussed or revised.

- What did the authors consider as dysplasias? Ductal or lobular hyperplasia, cysts, duct ectasia, focal fibrosis and gynecomastia can all be considered as dysplasias, therefore this classification should be cleared.

- The number of dysplasia samples is so small that statistically valid conclusions cannot be drawn.

- What areas did the authors consider in the evaluation of carcinoma in benign tumors? This is an important information seen that this type of tumor can have benign, malignant, in situ or invasive areas.

Reviewer: 2
Comments to the Author
The aim of this manuscript is to investigate progesterone receptor and its isoforms in formalin-fixed and paraffin embedded tissue samples from mammary dysplatic and neoplastic tissues by one-step SYBR Green RT-q-PCR. The results of molecular analysis were compared with immunohistochemistry labeling. The topic of the manuscript is innovative and add important knowledge to the study of canine mammary tumors. The results obtained are partially in contrast with previous studies carried out in breast cancer, but the Authors argue this inconsistency in the "Discussion section". The text is written in a sufficiently understandable English, for my skills. Perhaps, Figure 2 and 3 could be omitted because the same data are presented within the text. In conclusion I believe that this manuscript could be accepted for publication in veterinary Pathology after a minor revision.

Comments from the photo editor (dmg):
Fig 1: The associate editor is asking you to delete this photo. However, if for whatever reason you end up keeping it, you will need to change the mode to grayscale. In Photoshop, go to the IMAGE dropdown menu and select MODE and then GRAYSCALE. Also, please spell out FFPE completely the first time you use it in a legend.
Fig 2: Please spell out PR completely the first time you use it in a legend.
Fig 4: The image is too small. Please increase the width to a full two columns (180 mm) and then, AFTER resizing, please place the figure number in regular 14 point Arial font.
Fig 5 and 6: Please use the standard format for legends as given in the instructions to authors and copied below for your convenience. "Animal identification" in this case means species. Make sure to note the counterstain at the end of the legend, and please delete mention of magnifications.
FIGURE LEGEND RULES

Fig. 1. Organ or tissue; animal identification, case No. 1. Sentence description of the change that is visible
in the figure. Complete staining method with names of stains and counterstains

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Please plate figs 5 and 6 next to each other, forming a row 180 mm wide. Please don't place a white line separating the photos, that is added at press.
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debbie.gillette@att.net

Associate Editor: Kiupel, Matti
Comments to the Author:
Dear authors,

please clarify the classification of the lesions as suggested by reviewer 1 and specify which areas were selected how for Rt-qPCR. The manuscript would also benefit from a thorough review by a native English speaker. The authors often use a passive sentence structure that make the reading awkward and unnecessarily lengthens the manuscript, e.g." Results

showed the feasibility of analysis .... Why not using an active sentence structure and say: "Analysis of progesteron receptors by RT-qPCR was successful in routinely formalin fixed paraffin embedded samples." Figure 1 can be deleted.

Date Sent: 09-Aug-2013

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3.3. La isoforma A del receptor de progesterona puede regular los efectos del tratamiento neoadyuvante con aglepristona en el carcinoma de mama canino.

Journal of Veterinary Internal Medicine

JOURNAL
OF VETERINARY INTERNAL MEDICINE

Progesterone receptor isoforms A may regulate the effects of neoadjuvant aglepristone in canine mammary carcinoma

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Keywords (You can enter any word desired, do not have to use from the list):	Clinical Trials, Molecular biology-general < Molecular biology, PCR assays < Molecular biology, Oncology-treatment < Oncology

BRIEF COMMUNICATION

Progesterone receptor isoforms A may regulate the effects of neoadjuvant aglepristone in canine mammary carcinoma

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[1] Dept. Comparative Pathology, Veterinary Faculty, University of Córdoba, Córdoba, Spain.
[2] Institute of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Short title: Aglepristone in canine mammary carcinoma

Key words: hormone therapy, mammary tumors, RT-qPCR, dog.

List of abbreviations used in the manuscript:

PR: Progesterone receptors

PRA: Progesterone receptor isoform A

PRB: Progesterone receptor isoform B

RT-qPCR: Real time quantitative polymerase chain reaction

mRNA: Messenger ribonucleic acid

FFPE: Formalin-fixed, paraffin embedded

A_{260}: Absorbance at 260 nm

HPTR1: Hypoxanthine phosphoribosyl-transferase 1

RPL32: Canine ribosomal protein L32

IHC: Immunohistochemistry

ABC: Avidin-biotin-peroxidase complex

BLAST: Basic Local Alignment Search Tool

PI: Proliferation index

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This work was done at the Department of Comparative Pathology of the Veterinary Faculty of the University of Córdoba (Córdoba, Spain) and the Institute of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

This work was supported by grant number AGL2011-25553, Spanish Ministry of Education.

Abstract

Background: Progesterone receptors (PR) play a key role in the development of canine mammary tumors and there are recent evidences which focus on PR as possible therapeutic targets using antiprogestins. Cloning and sequencing of the PR gene has shown that the canine PR has two isoforms, PRA and PRB, transcribed from a single gene. Experimental studies in human breast cancer suggest that the differential expression of PR isoforms has implications for hormone therapy responsiveness.

Objetives: The aim of the present study was to study the relationship between the effects of the antiprogestin aglepristone in cell proliferation and mRNA expression levels of PRA and PRB in canine mammary carcinomas.

Animals: Twenty seven no spayed female dogs with a histological diagnosis of mammary carcinoma were treated with 20 mg/Kg aglepristone (22) or vehicle (5) twice before surgery.

Methods: Formalin fixed, paraffin embedded tissue samples taken before and after treatment were used to analyze PR, PRA and PRB by RT-qPCR and Ki67 antigen labeling by immunohistochemistry.

Results: Both PR and PRA mRNA expression levels decreased after treatment with aglepristone. Further, a significant decrease in proliferation index (percentage of Ki67-labeled cells) was observed in aglepristone-treated tumors which expressed PR and PRA.

Conclusions and clinical importance: These findings suggest that the antiproliferative effects of aglepristone in canine mammary carcinoma are mediated by PRA.

Epidemiological and clinical data indicate that progesterone has proliferative effects on normal and neoplastic canine mammary epithelium[1]. At time of diagnosis, some two thirds of canine mammary carcinomas have progesterone receptor (PR) labeling by immunohistochemistry[2] (IHC) and neoadjuvant treatment of these tumors with the antiprogestin aglepristone decreased cell proliferation on a PR-labeling dependent basis[3]. Aglepristone is currently used in clinical practice for induction of abortion and pyometra as well as for proliferative progesterone-dependent diseases such as mammary fibroadenomatous hyperplasia in queens and vaginal tumors in bitches.

Similarly to human PR, canine PR exists as two isoforms known as PR isoform A (PRA) and PR isoform B (PRB), which are transcribed from a single gene under the control of distinct promoters[4]. Under physiological conditions, tissue samples from normal human breast express both PRA and PRB at equimolar levels[5]. However, an altered PRA/PRB ratio is often associated with breast carcinogenesis with a predominance of PRA over PRB in benign and malignant human breast tumors[5]. In the canine species, there are controversial results due to the paucity of studies on the issue and the limited number of samples analyzed. Western blot analysis of two canine mammary glands in metoestrus, two in anoestrus and two after prolonged treatment with progestins showed that PRA was equimolar or more prominently present than PRB in most of the samples whereas predominance of PRB was found in one case only[4]. Further, predominant staining for PRA with less intense staining of PRB was observed in two normal, three hyperplasias and three carcinomas of the canine mammary gland by Western blot analysis also[6].

Despite their structural similarities, human PRA and PRB have demonstrated to have different functions since they regulate different subset of genes[7]. In human breast cancer, carcinomas with higher levels of PRA than PRB were inhibited by

antiprogestins whereas carcinomas with high levels of PRB did no respond to endocrine treatment[7]. Accordingly, it has been suggested that the evaluation of PR isoforms A and B may be important in prognosis and therapeutic decisions[5]. We have previously shown that 1) neoadjuvant treatment with aglepristone decreases cell proliferation in carcinomas with immunohistochemical PR labeling[3] and 2) PRA and PRB mRNA expression can be analyzed in formalin fixed, paraffin-embedded tissue samples of the canine mammary gland by RT-qPCR[a]. The aim of the present study was to study the relationship between the effects of aglepristone in the proliferation index and the mRNA expression levels of PRA and PRB in canine mammary carcinomas.

Materials and methods

Tissues

Formalin-fixed paraffin embedded (FFPE) tissue samples of 27 canine mammary carcinomas were used. These samples were taken from a series of 27 no spayed female dogs that received 2 subcutaneous injections of aglepristone[b] (22 dogs) or vehicle (5 dogs) on days 1 and day 7 before surgery at day 15[3]. To analyze the effects of aglepristone, a core biopsy was taken on day 1 before treatment and at day 15 through surgery[3]. Fixation time ranged between 24 and 48 hours, and paraffin blocks were stored at 4 ºC. Histological types of tumors included 14 complex carcinomas, 6 simple carcinomas, 5 carcinomas in benign tumor, 1 carcinosarcoma and 1 squamous cell carcinoma.

RT-qPCR analysis

For RNA isolation, the RNase FFPE kit[c] was used according to manufacturers' recommendations. RNA yields and purity were determined by the spectrophotometric

absorbance at 260 nm (A_{260}) measured using the NanoDrop® ND-1000 spectrophotometer[d] and stored at -80 ºC until using. Then, RNA was amplified using the LightCycler® 480 Real-Time PCR System. One-step RT-qPCR was performed using the QuantiFast® SYBR® Green RT-PCR[c] and according to the previously described protocol[a]. Canine-specific primers for PR gene were designed specifically to target the region of canine isoform B and the common region of both isoforms (total PR) using Primer3Plus.The expression of PR was normalized relative to two canine housekeeping genes hypoxanthine phosphoribosyl-trasnferase 1 (HPTR1) and canine ribosomal protein L32 (RPL32). Table 1 summarizes the sequences of the forward and reverse primers for PR gene and housekeeping genes. Reverse transcription negative controls and non template controls were included.

RT-qPCR data was presented as $2^{-\Delta Ct}$ where $\Delta Ct = Ct_{TARGET} - Ct_{RPL32/HPTR1,}$ and $Ct_{RPL32/HPTR1}$ is the average of the Ct values of the two housekeeping genes for each sample. The amount of PRA was calculated by subtracting the relative amount of PRB from that of the total PR according to other studies in human medicine[8]. The PR positive-status cut-off was established at 0.04 values since it represented the best concordance with IHC results (Kappa index 0.06, Figure 1).

Immunohistochemical study

1. PR labeling

The commercial mouse monoclonal anti-human PR antibody[e] (clone 10A9) isotype IgG_2 diluted 1:500 and the ABC method[f] were applied as described[3]. This antibody recognizes the recombinant hormone-binding domain of human PR located on the C-terminal region which is a common region of PRA and PRB. BLAST analysis demonstrated that the epitope detected by this clone (extreme C-terminus of the human

PR) is identical to the corresponding part of the canine PR which may be used in the canine species as well http://blast.ncbi.nlm.nih.gov/. PR labeling was evaluated quantitatively in digital pictures of tissue sections taken at a 40x magnification from 4 randomly selected neighboring, non overlapping fields per sample. The number of positive and negative cells was counted with ImageJ software[g]. A minimum of 1000 tumor cells were counted per case. PR labeling was expressed as the percentage of positive cells related to the total number of cells. Classification of PR status of carcinomas by IHC was done using a cut-off of 10%.

2. Proliferation index (PI)

Proliferation index was previously analyzed by IHC in all samples (Guil-Luna et al., 2011). The monoclonal mouse anti-human Ki67 antigen (clone MIB-1) isotype IgG_1[h] diluted 1:75 and the ABC method was applied as described[3]. Positive and negative tumor cells were counted with a pen tablet[i]. PI was expressed as the percentage of positive tumor cells related to the total number of cells. A minimum of 1000 cells were counted per case.

Statistical analysis

For statistical data evaluation the software GraphPad PRISM 5 version 5.01[j] was used. The values were evaluated for normality analysis using the D'Agostino-Pearson normality test. Differences between the means of RU534-treated tumors before and after treatment were assessed by paired t-tests or Wilcoxon test. Concordance degree between RT-qPCR and IHC methods was estimated by Cohen´s κ coefficient. A P value < 0.05 was regarded as statistically significant.

Results

PR labeling

Sixty percent of control and 59.1% of experimental tumors were classified as PR positive tumors at the time of diagnosis[3]. Before treatment, complex carcinomas and carcinomas in benign tumor were PR-positive (86% each) more frequently than simple carcinomas (33%). After treatment, a decrease in the number of PR-positive tumors was observed in aglepristone-treated tumors exclusively. On the contrary, a single case of PR-negative tumor at day 1 was found to be PR-positive at day 15[3].

PR, PRA and PRB mRNA expression levels

Before treatment, PR mRNA values ranged from 0 to 0.27 while figures after treatment ranged from 0.01 to 0.15. According to RT-qPCR results, 60% of control and 55% of experimental tumors were considered to be PR-positive before treatment. Complex carcinomas and carcinomas in benign tumor had similar PR mRNA (0.075 and 0.069, respectively) whereas simple carcinomas showed the lowest levels ($p = 0.002$ and $p = 0.04$, respectively). A significant decrease of PR mRNA was observed after treatment in PR-positive aglepristone-treated tumors exclusively ($p = 0.001$) (Table 2) whereas control and PR-negative aglepristone-treated tumors did not show any significant change.

Before treatment, PRA mRNA values ranged from 0 to 0.21 and PRB mRNA values from 0 to 0.06. Corresponding figures after treatment were 0.01 to 0.11 for PRA and 0 to 0.06 for PRB. Some 50% of samples had 3 to 10 folds higher expression of PRA mRNA than PRB mRNA at day 1, and this expression of PRA was significantly higher than the expression of PRB in 82 % of control and experimental carcinomas ($P = 0.003$). At day 15, a significant decrease of PRA expression was observed in PRA-positive aglepristone-treated tumors exclusively ($p=0.001$) (Table 3) whereas PRA-

negative or control tumors did not show significant changes. PRB expression was not affected by aglepristone treatment in any case (Table 4).

Proliferation index and PR, PRA and PRB expression

A significant decrease in PI after aglepristone treatment was observed in PR- and PRA-positive tumors of the treated group exclusively ($P = 0.007$, $P = 0.01$, respectively) (Figure 2, Figure 3). Nearly 70% of those cases had a PI reduction of $\geq 20\%$. No significant changes in the PI were observed when PRB expression was taken into account (Figure 4).

Discussion

Results of this study showed that the proliferation index of PR- and PRA-positive tumors decreased significantly after aglepristone treatment. These findings confirm previous data indicating a direct relationship between PR labeling and the antiproliferative effect of aglepristone in canine mammary carcinoma[3] and suggest that this effect is mediated by PRA.

In normal human breast, PRA and PRB are generally expressed at similar levels but in breast cancers, their ratio is deregulated with a predominance of PRA over PRB[5]. In this study, canine mammary carcinomas showed higher PRA than PRB expression levels. These results are in agreement with those reported in human breast cancer as well as in the few samples of mammary tumors studied in the canine species[4,6].

In women, more than 70% of breast cancers express estrogen receptors and PR and are thus eligible for adjuvant endocrine therapy[5]. This adjuvant therapy is designed to target the ER by using ER modulators or by inhibiting the endogenous synthesis of 17β-

estradiol with aromatase inhibitors. Some recent investigations point to PR as a therapeutic target as well[5]. In this regard, different experimental models of breast cancer have demonstrated an inhibitory effect of antiprogestins either alone or together with antiestrogens[9]. In female dogs, some 75% of carcinomas express PR by immunohistochemistry[2] but, contrary to the situation in women, adjuvant endocrine treatment aimed to block the PR is not used.

In the present study, tumors with PR and PRA at day 1 presented a significant decrease in the proliferation index after aglepristone treatment. Results concerning PR expression levels are in agreement with those previously reported in which PR status was determined by immunohistochemical labeling[3]. Moreover, a high concordance was found between PR expression by RT-qPCR and IHC, as described in human studies[10]. However, results concerning the relationship between PR isoforms and proliferation of canine mammary carcinomas are novelty. In human breast cancer, several studies have associated the inhibitory effect of antiprogestins to PRA but not to PRB expression[7]. In these studies, a down regulation of PRA was observed, as in canine carcinomas, and suggested to be responsible for the antiproliferative effects of antiprogestins. The fact that we have not observed significant changes regarding PI and PRB mRNA in tumors analyzed suggests that PRA mediates the antiproliferative effect of aglepristone and highlights the differential roles of PRA and PRB in the canine mammary gland.

In conclusion, results have shown that neoadjuvant treatment of canine mammary carcinomas with the antiprogestin aglepristone reduced cell proliferation in those tumors considered PR-positive by the RT-qPCR method. They have also shown the same effect of aglepristone in PRA-positive tumors, suggesting that the antiproliferative effect of aglepristone in canine mammary carcinoma is most probably mediated by PRA.

Footnotes

[a] Guil-Luna S, Stenvang J, Brünner N, Sánchez-Céspedes R, Millán Y, Gómez-Laguna J, Martín de las Mulas J. Detection of progesterone receptor by qPCR analysis from formalin-fixed paraffin embedded canine mammary tissues. Abstract meeting. European Association of Veterinary Pathology, 2012.

[b] Alizine, Virbac, France.

[c] Qiagen, Copenhagen, Denmark.

[d] Thermo Scientific, Wilmington, DE, USA.

[e] Immunotech, Marseille, France.

[f] Vector, Burlingame, CA, USA.

[g] ImageJ 1.43, National Institute of Health, Maryland, USA.

[h] Dako, Barcelona, Spain.

[i] Volito 2, Wacom Europe GmbH, Krefeld, Germany.

[j] GraphPad Software Inc, San Diego, CA, USA.

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6. Gracanin A, Gier J, Zegers K, Bominaar M et al. Progesterone receptor isoforms in the mammary gland of cats and dogs. Reproduction in domestic animals 2012;47:313.317.

7. Wargon V, Helguero LA, Bolado J et al. Reversal of antiprogestin resistance and progesterone receptor isoform ratio in acquired resistan mammary carcinomas. Breast cancer research and treatment 2009;116:449-460.

8. Hayashi A, Tanabe A, Kawabe S et al. Dienogest increases the progesterone receptor isoform B/A ratio in patients with ovarian endometriosis. Journal Ovarian Research 2012;5:1-8.

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10. Oda M, Arihiro K, Kataoka T et al. Comparison of immunohistochemistry assays and real-time reverse transcription-polymerase chain reaction for analyzing hormone receptor status in human breast carcinoma. Pathol Int. 2010;60:305-315.

313 **Tables**

314

315 **Table 1.- Primers sequences for RT-qPCR amplification**

	Primer Forward	Primer Reverse
PR	5'-GGCTTGCCGCAGGTGTACCA-3'	5'- ACTGTGGGCTCTGGCTGGCA-3'
PRB	5´-CCCGGGCGGATCCGAGACT-3´	5´-ACTGTGGGCTCTGGCTGGCA-3
HPTR1	5'-TGCAGACTTTGCTTTCCTTGGTCA-3'	5'-TCGAGGGGTCCTTTTCACCAGCA -3'
RPL32	5'-GGCTGCCCTCAGACCTCTGGT -3'	5'-TCGGTCTGACTGGTGCCGGA -3'

316 PR: Progesterone receptor; PRB: Progesterone receptor isoform B;

317 HPTR1:Hypoxanthine phosphoribosyl-transferase 1; RPL32: Canine ribosomal protein

318 L32

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320

321

322

Table 2.- Total PR expression at day 1 and at day 15 according to PR status by RT-qPCR at day 1.

	PR status at day 1	Mean ± SD of PR expression (2^-ΔCt)	
		Day 1	Day 15
Control group	PR+	0.07 ± 0.02	0.08 ± 0.06
	PR-	0.02 ± 0.00	0.03 ± 0.00
Treated group	PR+	0.10 ± 0.07	0.04 ± 0.03*
	PR-	0.02 ± 0.01	0.05 ± 0.05

PR: Progesterone receptor; SD: Standard deviation; PR+: Progesterone receptor positive; PR-: Progesterone receptor negative.

Table 3.- PRA expression at day 1 and at day 15 according to PRA status by RT-qPCR at day 1.

	PRA status at day 1	Mean ± SD of PRA expression (2^Δ-Ct)	
		Day 1	Day 15
Control group	PRA+	0.05 ± 0.02	0.08 ± 0.06
	PRA-	0.02 ± 0.01	0.03 ± 0.00
Treated group	PRA+	0.09 ± 0.05	0.05 ± 0.02*
	PRA-	0.01 ± 0.01	0.03 ± 0.04

PRA: Progesterone receptor isoform A; SD: Standard deviation; PRA+: Progesterone receptor isoform A positive; PRA-: Progesterone receptor isoform A negative.

Table 4.- PRB expression at day 1 and at day 15 according to PRB status by RT-qPCR at day 1.

		Mean ± SD of PRB expression (2^-ΔCt)	
	PRB status at day 1	Day 1	Day 15
Control group	PRB+	0.018 ± 0.01	0.015 ± 0.02
	PRB-	0.007 ± 0.00	0.007 ± 0.001
Treated group	PRB+	0.018 ± 0.01	0.015 ± 0.01
	PRB-	0.006 ± 0.002	0.006 ± 0.003

PRB: Progesterone receptor isoform B; SD: Standard deviation; PRB+: Progesterone receptor isoform B positive; PRB-: Progesterone receptor isoform B negative.

Figure legends

Figure 1.- Comparison of RT-qPCR values versus immunohistochemical assessment in the 27 samples. The Yaxis shows PR expression values by RT-qPCR and X axis the PR (+) and (-) samples according IHC. The cut-off for PR (+) status by RT-qPCR is indicated by the horizontal broken line. Black points show discrepancy between both methods.

Figure 2.- Proliferation index. Effect of aglepristone treatment on the Ki67 percentage on PR-positive treated (A), PR-negative treated (B), PR-positive control (C) and PR-negative control (D) canine mammary carcinomas at day 1 and day 15. * $P < 0.05$.

Figure 3.- Proliferation index. Effect of aglepristone treatment on the Ki67 percentage on PRA-positive RU534-treated (A), PRA-negative RU534-treated (B), PRA-positive control (C) and PRA-negative control (D) canine mammary carcinomas at day 1 and day 15. * $P < 0.05$.

Figure 4.- Proliferation index. Effect of aglepristone treatment on the Ki67 percentage on PRB-positive treated (A), PRB-negative treated (B), PRB-positive control (C) and PRB-negative control (D) canine mammary carcinomas at day 1 and day 15. * $P < 0.05$.

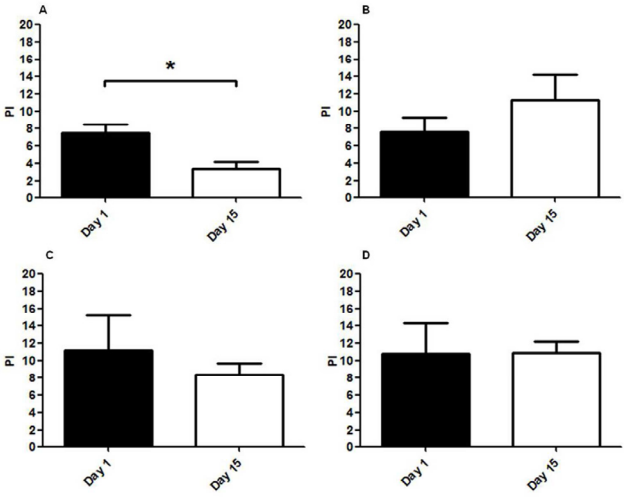

Figure 2.- Proliferation index. Effect of aglepristone treatment on the Ki67 percentage on PR-positive treated (A), PR-negative treated (B), PR-positive control (C) and PR-negative control (D) canine mammary carcinomas at day 1 and day 15. * P < 0.05.
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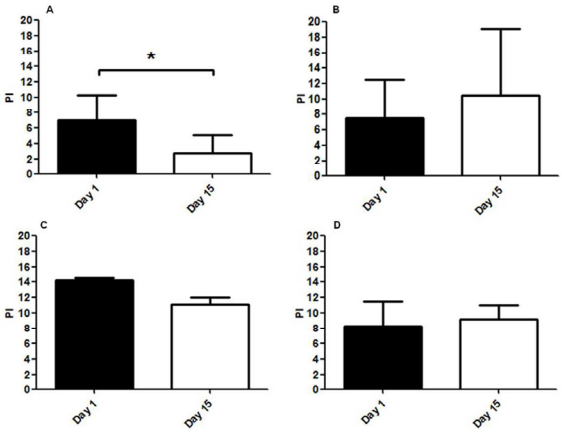

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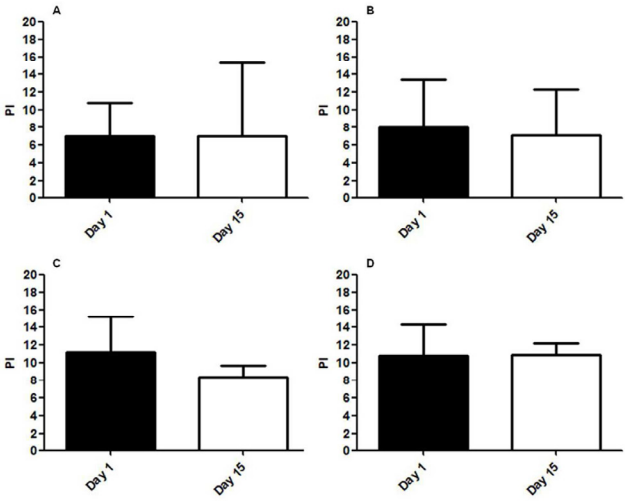

Figure 4.- Proliferation index. Effect of aglepristone treatment on the Ki67 percentage on PRB-positive treated (A), PRB-negative treated (B), PRB-positive control (C) and PRB-negative control (D) canine mammary carcinomas at day 1 and day 15. * P < 0.05.
80x63mm (300 x 300 DPI)

3.4. Los antiprogestágenos mifepristona y onapristona reducen la proliferación celular en las células de carcinoma de mama canino CMT-U27

The antiprogestins mifepristone and onapristone reduce cell proliferation in the canine mammary carcinoma cell line CMT-U27

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Keywords: progesterone; antiprogestins; canine mammary carcinoma cell line.

Introduction

Canine mammary tumours (CMTs) represent nearly half of all tumours in female dogs and some 50% have malignant behaviour (Sorenmo et al. 2013). Epidemiologic, clinical and experimental data indicate that CMTs are hormone-dependent, that is, are strongly influenced by ovarian hormones, mainly estrogens and progesterone (P) (Sorenmo et al. 2013). Prolonged exposure to high concentrations of progesterone during the comparatively long luteal phase of the oestrous cycle is suspected to be the key event in canine mammary tumorigenesis (Evans et al. 1969). Ovariectomized female dogs when exposed to progestins for 6-12 months developed mammary gland hyperplasias and/or benign tumours (Selman et al. 1994; Bhatti et al. 2007). Further, occurrence of mammary carcinomas has been reported in dogs upon administration of high doses of progestins (Kwapien et al. 1980). Studies of gene expression profiles indicate a strong cell proliferation inducing effect of progestins in canine mammary hyperplasia and carcinoma (Rao et al. 2009). In women with breast cancer, hormone therapy is mostly focused on the blockade of oestrogen receptors as well as on the use of aromatase inhibitors because these receptors are expressed in approximately 70% of all cases (Knoop et al. 2007; Lanari et al. 2012). In the dog, two-thirds of mammary carcinomas express P receptors (PR) (Geraldes et al. 2000; Martín de las Mulas et al. 2005; Chang et al. 2009) with differences in expression level among different histological subtypes. Thus, luminal epithelial cell-type or *simple epithelial* carcinomas (Misdorp et al. 1999) have lower PR expression

than tumours composed of luminal epithelial and myoepithelial cells either alone or combined with mesenchymal components (the so-called *complex* and *mixed* carcinomas, respectively) (Misdorp et al. 1999; Martín de las Mulas et al. 2005). *Simple epithelial* carcinomas have shorter disease free periods after surgery than their *complex* and *mixed* counterparts (Martín de las Mulas et al. 2005) and may thus be the histological subtype that benefits most from the use of endocrine therapy. Recently, neoadjuvant administration of the antiprogestin aglepristone (RU534) to non-spayed female dogs with spontaneous mammary carcinomas was shown to reduce tumour cell proliferation on a PR-expression basis (Guil-Luna et al. 2011). In that work, the highest decrease in cell proliferation was observed in *simple epithelial* carcinomas. The aim of the present study was to analyze the effects of the RU534 analogue mifepristone (RU486) and the antiprogestin onapristone (ZK299) on a PR-positive canine mammary carcinoma cell line isolated from a *simple epithelial* carcinoma.

Materials and Methods

Cell line

The CMT-U27 cell line was established from a canine simple mammary carcinoma and has been given an account previously (Hellmén 1992; Misdorp et al. 1999; Król et al. 2009, 2010; Pawlowski et al. 2011). RU486 and ZK299 were kindly provided by Exelgyn, Paris (France) and Shering, Berlin (Germany), respectively.

Cell culture

The cell line was cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis,

MO, USA), supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), penicillin-streptomycin (50 iU mL-1) and kept at 37 ºC in 5% CO2 atmosphere in 75 cm2 culture flasks to 80% confluence. For the experiments, cells were grown in phenol red-free and steroid-deficient RPMI 1640 medium with antibiotics but without FBS supplemented.

Treatment of cells

Initially, the cells were cultured in complete RPMI medium without and with 0.01% ethanol for 24, 48 hours respectively. No significant differences on cell number were observed. Thus, the experiments were carried out with 0.01% ethanol supplement. CMT-U27 cells were treated with RU486 and ZK299 in absolute ethanol (final concentration 0.01%) at 10^{-6}M (Lin et al. 2001; Formby et al. 1999; Neubauer et al. 2011) during 24h and 48h each. Control cells were treated with 0.01 % ethanol for 24 and 48 hours. All experiments were performed in triplicate.

Cell proliferation and viability assay

The colorimetric WST-8 assay (Promokine, Sweden) based in the WST-8 reduction by cellular dehydrogenases to an orange formazan product which is directly proportional to the number of living cells was used. Following manufacturer's recommendations, the optimal number of cells per well ensuring exponential cell growth for the entire period of the assay was first established at 2500 cells/well (Fig. 1). Then, cells were seeded in 96-well plates at a density of 2500 cells per well. After 24h attachment and three times rinsing in phosphate buffered saline, the medium was changed for phenol-red free steroid-deficient RPMI 1640 medium and the drugs (RU486 and ZK299) were added. After incubations, 10µl of

CCVK-I solution was added and measures of the absorbance at 450 nm were performed using a Multiscan Ascent microplate reader. Each sample was analysed in triplicate, and the mean value of absorbance was used as the final result.

PR expression immunocytochemical assay

Cultured cells were fixed in 4% formalin and embedded in paraffin using cell culture block technique (Li et al. 2005; Andersson et al. 2006; Mote et al. 2001). The monoclonal mouse PR antibody (clone 10A9) isotype IgG_2 (Immunotech, Marseille, France) diluted 1:400 was used for the detection of PR expression. A commercial diluent (Dako, Barcelona, Spain) was used. Heat induced antigen retrieval in a water bath at 95-99 ºC with 0.01M citrate buffer (pH 6.0) for 25 min was used. After cooling down at room temperature for about 30 min, slides were covered with 10% normal goat serum in PBS for 30 min before incubation with the primary antibody for 18 hours at 4 ºC. The avidin-biotin-peroxidase complex (Vector Laboratories; Burlingame, CA, USA) was applied for 1 hour at room temperature. The chromogen, 3,3-diaminobenzidine tetra-hydrochloride (Sigma, Saint Louis, USA) diluted 0.035% in 0.05 M Tris containing 0.3% of hydrogen peroxide was applied to the slides for 1 min at 20-22 ºC. For negative controls, the primary antibody was replaced by mouse IgG_2 (Dako, Barcelona, Spain). As positive control, formalin-fixed, paraffin embedded tissue samples of canine normal mammary gland were used.

The number of positive and negative cells was counted at a magnification of 40x by two different pathologists to ensure uniformity. All

cells present in each slide (5 slides for each with an average of 1500 cells/slide) were counted. PR expression was expressed as the percentage of positive cells related to the total number of cells.

Statistical analysis

Statistical analysis was carried out by the GraphPad Software 3.05 (San Diego, CA). The values were evaluated for approximate normality of distribution by the Kolmogorov-Smirnov test. Differences between treatments were assessed by Mann-Whitney test. Results were expressed as mean ± SD. A $p<0.05$ was regarded as statistically significant.

Results

Cell proliferation and viability

RU486 ($p<0.05$) and ZK299 ($p<0.05$) reduced the number of viable cells at 24h (Fig. 2).

PR expression

Immunoreactive cells exhibited a nuclear staining pattern (Fig. 3). Twenty five percent of CMT-U27 control cells expressed PR. This figure was similar in all groups of RU486-treated cells but ZK299 treatment significantly reduced PR expression in tumour cells at 24h of incubation ($p<0.05$) (Fig. 4).

Discussion

Present results show that treatment of CMT-U27 cells with 10^{-6}M RU486 or 10^{-6}M ZK299 reduced the number of tumour cells. Results also show that the number of PR-positive cells decreased after ZK299 treatment

exclusively. These findings show that this CMT-U27 cell line is sensible to the effects of antiprogestins and may be useful to analyze the role of these drugs in canine mammary tumours.

The CMT-U27 cell line has a high growth rate and anti-apoptotic potential associated with enhanced expression of genes involved in Ca^{2+} signalling pathway and growth hormone cellular pathway (Król et al. 2009). It was isolated from a *simple epithelial* carcinoma, the histological subtype with the shortest disease free period after surgery (Misdorp et al. 1999; Martín de las Mulas et al. 2005). For this reason, *simple epithelial* carcinomas may be suitable targets for endocrine therapy in female dogs. In vivo, these tumours are known to have a low expression level of PR as in the present case where some 25% of the CMT-U27 cells were labelled with the PR10A5 anti-PR antibody (Geraldes et al. 2000; Martín de las Mulas et al. 2005; Chang et al. 2009). In spite of this fact, PR-positive *simple epithelial* carcinomas not only responded to the antiproliferative effect of aglepristone *in vivo* but showed a decrease in MIB-1 index higher than complex and mixed carcinomas (Guil-Luna et al. 2011).

RU486 and ZK299 are antiprogestins that compete with P hormone binding at the receptor level and have demonstrated to have powerful effects on growth inhibition of mammary tumors and their metastasis in rodents and human breast cancer cell lines (Horwitz et al. 1992; Tieszen et al. 2011).

RU486 is a type II, partial PR antagonist that promotes PR binding to DNA (Lanari et al. 2012). In CMT-U27 cells, RU486 reduced the number of viable cells at 24 h of incubation. This finding is consistent with previous reports demonstrating a cytotoxic effect of RU486 in PR-positive human breast cancer cell lines T47D and MCF-7 (Bardon et al. 1987; Gaddy et al. 2004; Tieszen et al. 2011). It remains unclear whether the mechanism through which RU486 induces cytotoxicity in tumour cells is mediated by PR or not. In this study, we observed that RU486 had no effect on PR expression in accordance with previous findings (Hurd et al. 1999; Navo et al. 2008). Explanations for this finding are speculative and include 1) the failure of RU486 to induce the structural changes in the receptors that lead to their down regulation by degradation through an ubiquitin-proteasome pathway once the antagonist-PR complex is created (Hurd et al. 1999; Zhang et al. 2007) and 2) the PR-independent cytotoxic activity of RU486 (Liang et al. 2003; Tieszen et al. 2011). The latter explanation is very interesting because the capacity of RU486 to decrease cell viability coupled with its lack of effect on PR expression suggests that sequential hormone therapy could be administered without any loss of efficacy.

ZK299 is a type I, full PR antagonist, unable to induce PR binding to P response elements or promote DNA-dependent phosphorylation. Although it has been attributed a 10-fold lower affinity of ZK299 for PR than RU486 (Hurd et al. 1999), our present results show that a concentration of 10-6M ZK299 has substantial effect on the CMT-U27 cells. Thus, ZK299

decreased both cell viability and the number of viable PR-positive cells. Numerous reports have found ZK299 to reduce cell proliferation in PR positive breast cancer cell lines (Classen et al. 1993; Iwasaki et al. 1999) and to induce quantitative alterations of PR in breast cancer cells (Hurd et al. 1999) which suggests that down regulation of PR might play a role in the effects of this PR antagonist (Hurd et al. 1999). The possibility exists that complete occupation of available PR by "pure" progestin/ antiprogestins is necessary for PR down regulation. Again, the antagonist-PR complex may have induced structural changes in the receptors that lead to their degradation through an ubiquitin-proteasome pathway (Hurd et al. 1999; Zhang et al. 2007). Our present results suggest that both RU486 and ZK299 induce a decrease in the number of viable CMT-U27 tumour cells with different effect on PR expression. The fact that just 25% of the untreated CMT-U27 cells expressed PR might reflect some underlying heterogeneity in this cell population and clonal expansion is likely present *in vitro*. For further experimental studies on antiprogestins, the number of studied cell lines should be expanded and preferably also include cells that lack PR (Hellmén et al. 2000).

In conclusion, the canine mammary carcinoma cell line CMT-U27 is sensible to the effects of antiprogestins and may serve to further explore the role of these drugs in canine mammary carcinomas.

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Figure legends

Fig. 1 Growth rate of CMT-U27 cell line in different initial cell concentration (range of 1250 to 20000 cells/well)

Fig. 2 Cell proliferation assay. Effect of mifepristone (RU486) and onapristone (ZK299) treatment on the viability of CMT-U27 cells at 24 and 48h of incubation. The results are expressed as mean±SD. * $p < 0.05$. Mann-Withney test

Fig. 3 PR expression by immunocytochemical assay. CMT-U27 cells show nuclear labelling with anti-PR antibody. ABC method. Bar 10µm

Fig. 4 PR expression related to cell treatment. Percentage of total PR positive cells in CMT-U27 cells with treated with mifepristone (RU486) and onapristone (ZK299) at 24 and 48h of incubation. The results are expressed as mean±SD. * $p < 0.05$. Mann-Withney test.

Figures

Figure 1

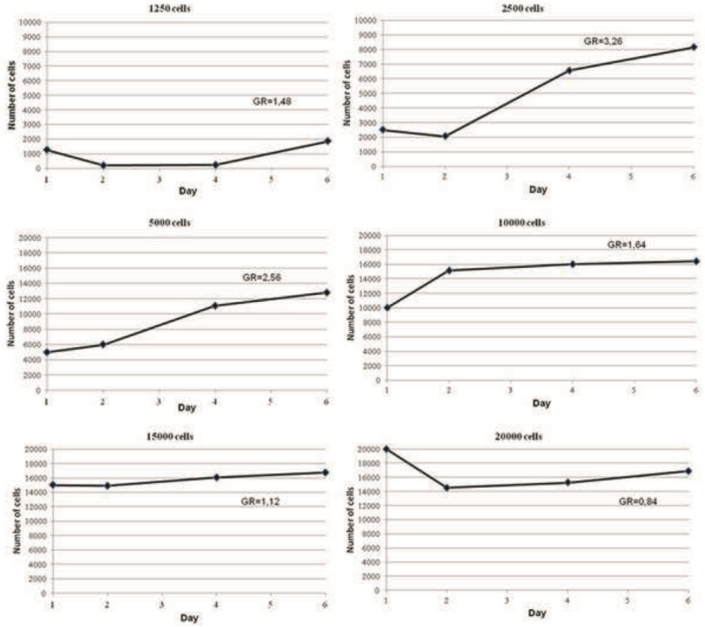


Figure 2

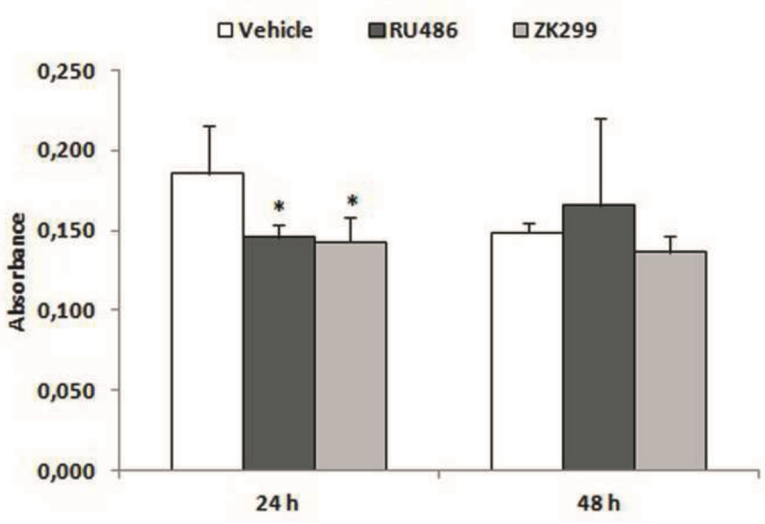


Figure 3

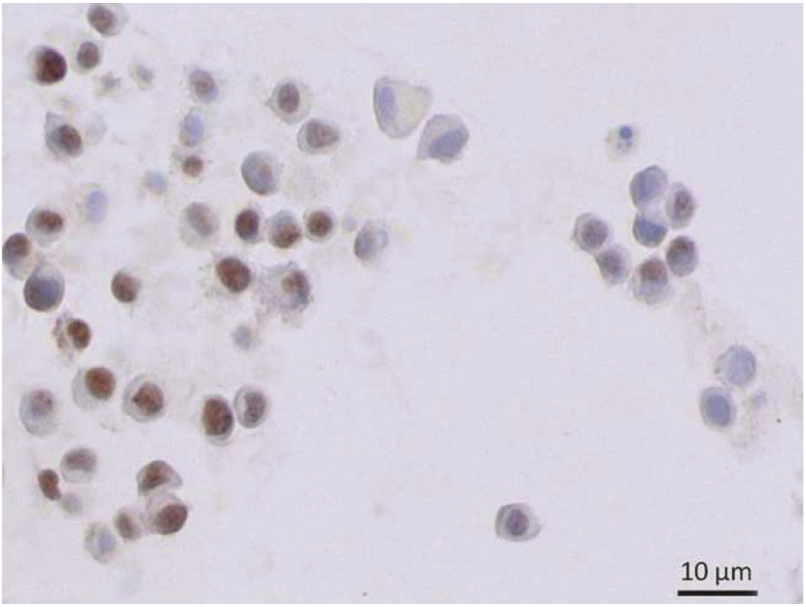
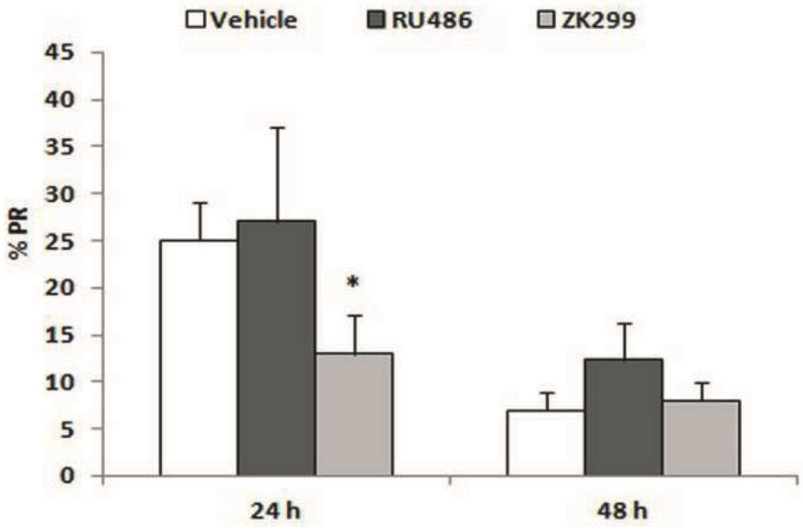


Figure 4



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4. Conclusiones
Conclusions

4. Conclusiones

1 El tratamiento neoadyuvante de perras con carcinoma de mama con el antiprogestágeno *aglepristona* disminuye la proliferación celular en los casos con expresión de receptores de progesterona analizados tanto con una técnica inmunohistoquímica como con la técnica cuantitativa de la RT-qPCR.

2 La disminución de la proliferación inducida por el tratamiento de las perras con el antiprogestágeno *aglepristona* se asocia a la expresión de la isoforma A del RP exclusivamente en carcinomas de mama canina.

3 El tratamiento neoadyuvante de perras con carcinoma de mama con el antiprogestágeno *aglepristona* disminuye la expresión del receptor de progesterona completo y de la isoforma A del receptor de progesterona exclusivamente.

4 Las muestras de tejido de la mama canina displásica y neoplásica procesadas rutinariamente para estudio histopatológico son válidas para analizar la expresión del ARNm del receptor de progesterona y de sus isoformas A y B mediante la técnica de RT-qPCR.

5 Las células de carcinoma de mama canino CMT-U27 tienen un nivel de expresión inmunohistoquímica de receptores de progesterona bajo que es característico de los carcinomas simples *in vivo*.

6 Las células de carcinoma de mama canino CMT-U27 son sensibles a los efectos de los antiprogestágenos mifepristona y la onapristona y pueden ser útiles para futuros estudios relacionados con el uso de estos fármacos en el cáncer de mama.

4. Conclusions

1. In female dogs with mammary carcinoma, neoadyuvant treatment with the antiprogestin *aglepristone* decreases cell proliferation in cases with progesterone receptor expression as determined by both immunohistochemistry and RT-qPCR.

2. The decrease in cell proliferation induced by *aglepristone* treatment in female dogs with mammary carcinoma is associated with the expression of isoform A of progesterone receptor exclusively.

3. In female dogs with mammary carcinoma, neoadyuvant treatment with the antiprogestin *aglepristone* decreases both total progesterone receptor and progesterone receptor isoform A expression.

4. Tissue samples of canine mammary dysplasias and neoplasias routinely processed for histopathological study are valid to analyze the mRNA expression of the progesterone receptor and their isoforms A and B by RT-qPCR.

5. Canine mammary carcinoma cell line CMT-U27 has a low level of progesterone receptor expression as measured by immunohistochemistry which is characteristic of simple carcinomas *in vivo*.

6. Canine mammary carcinoma CMT-U27 cells are sensitive to the effects of the antiprogestins mifepristone and onapristone and may be useful for future studies on the use of these drugs in mammary carcinoma.

5. Resumen
Summary

5. Resumen

Los tumores de mama en la especie canina representan casi la mitad de las neoplasias que afectan a la perra. Dada su elevada frecuencia, los tumores de mama caninos son importantes causas de morbilidad y mortalidad. Alrededor del 50% de los tumores mamarios diagnosticados son considerados malignos y por tanto, con una evolución clínica postquirúrgica desfavorable. Muchos de ellos tienen receptores de progesterona (RP), dato que evidencia la importancia de esta hormona en el desarrollo de la enfermedad. Sin embargo, a diferencia de lo que ocurre en la mujer con el receptor de estrógenos, no hay datos sobre los efectos de los antiprogestágenos en el tratamiento sistémico de las perras con cáncer de mama. En este trabajo hemos analizado los efectos in vivo e in vitro de los antiprogestágenos sobre la proliferación del carcinoma de mama canino con expresión de RP.

El estudio *in vivo* se realizó en 27 perras no castradas (22 experimentales y 5 controles) con tumores espontáneos tratadas con 2 dosis de 20 mg/Kg de aglepristona antes de la cirugía y observamos una disminución de la proliferación celular (índice Ki67, MIB-1, método ABC) en los tumores que tenían receptores de progesterona tanto con una técnica inmunohistoquímica (anticuerpo PR10A9, técnica ABC) como la técnica cuantitativa de reacción en cadena de la polimerasa en tiempo real (RTqPCR). Esta última técnica se estandarizó y aplicó por primera vez en la literatura sobre muestras de displasias y tumores de la mama canina procesadas rutinariamente para estudio histopatológico y

permitió analizar no solo la expresión del RP completo sino también de sus isoformas A y B. La isoforma A resultó ser la de expresión más frecuente en el carcinoma de mama canino y la que estaba asociada a la reducción de la proliferación celular en los animales tratados con aglepristona. Otro efecto del tratamiento in vivo fue la disminución de los RP en las muestras postratamiento tanto completo como de la isoforma A.

El estudio *in vitro* se realizó en la línea celular de carcinoma de mama canino CMT-U27 tratada con 10-6M de los antiprogestágenos mifepristona y onapristona durante 24 y 48 horas, y observamos una disminución de la proliferación celular (test de viabilidad WST-8) con ambos agentes a las 24 horas de tratamiento. También observamos que las células CMT-U27 tenían una expresión inmunohistoquímica baja (25%) de RP compatible con los niveles de expresión del tipo histológico del que fue aislada (carcinoma simple) y que la onapristona, pero no la mifepristona, disminuyeron los niveles de RP a las 24 horas. Los resultados de estos estudios indican que las células del carcinoma de mama canino que expresan RP son sensibles a los efectos antiproliferativos de los antiprogestágenos tanto *in vivo* como *in vitro*.

5. Summary

Canine mammary tumours account for nearly half of the neoplasms affecting the female dog. Given their high frequency, mammary tumours are important causes of morbidity and mortality. Some 50% of canine mammary tumours are diagnosed as malignant and therefore, are expected to have an unfavourable clinical course after surgery. Many mammary carcinomas have progesterone receptors, evidencing the importance of this hormone in the development of the disease. However, unlike what happens in human breast cancer with respect to oestrogen receptors, there are no data available concerning the role of antiprogestins in the systemic treatment of female dogs with mammary cancer. In this study we have analyzed the *in vivo* and *in vitro* effects of antiprogestins on the proliferation of canine mammary carcinomas with progesterone receptors.

The in vivo study was performed on 27 no spayed female dogs (22 experimental and 5 controls) with spontaneous tumours treated with 2 doses of 20 mg / Kg aglepristone before surgery. Results showed a decrease in cell proliferation (Ki67 index, MIB- 1 antibody, ABC method) in tumours expressing progesterone receptors as measured by immunohistochemistry (PR10A9 antibody, ABC technique) and by real time quantitative polymerase chain reaction (RTqPCR). The latter technique was standardized and applied for the first time in the literature in the dog by using tissue samples of mammary dysplasias and tumours routinely processed for histopathological examination. This technique allows the study of both the complete progesterone receptor as well as their isoforms

A and B. Results showed that isoform A was the most commonly expressed in carcinomas as well as to be the isoform associated with the reduction in cell proliferation after aglepristone treatment. Another in vivo effect of this treatment was the decrease of both the complete progesterone receptor and its isoform A.

The *in vitro* study was carried out with the canine mammary carcinoma cell line CMT-U27 treated with 10-6M of the antiprogestins onapristone and mifepristone for 24 and 48 hours. Results showed a decrease in cell proliferation (WST-viability test 8) with both drugs at 24 hours of treatment. CMT-U27 cells had a low immunohistochemical expression of progesterone receptors (25%) compatible with corresponding expression levels of the histological type it was isolated from (single carcinoma). Finally, onapristone, but not mifepristone, decreased progesterone receptor expression levels at 24 hours of treatment. Results of these studies suggest that canine mammary carcinoma cells expressing progesterone receptors are sensitive to the antiproliferative effects of antiprogestins both *in vivo* and *in vitro*.

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7. Lista de abreviaturas
List of abbreviations

7. Lista de abreviaturas

ABC: Complejo avidina-biotina-peroxidasa

ADN: Ácido desoxirribonucleico

ARNm: Ácido ribonucleico mensajero

BRCA1: gen "cáncer de mama 1"

BRCA2: gen "cáncer de mama 2"

C-erb-2: Receptor 2 del factor de crecimiento epidérmico humano

DBD: Dominio de unión a ADN

DCC: Dextran-coated charcoal

DMBA: Dimetilbenzantraceno

ESR1: Receptor de estrógenos 1

GH: Hormona de crecimiento

GnRH: Hormona liberadora de gonadotropinas

IHQ: Inmunohistoquímica

LBD: Dominio de unión a ligando

OMS: Organización Mundial de la Salud

P: Progesterona

P21: Proteína tumoral 21

P27: Proteína tumoral 27

P53: Proteína tumoral 53

RE: Receptor de estrógenos

RP: Receptor de progesterona

RPA: Isoforma A del receptor de progesterona

RPB: Isoforma B del receptor de progesterona

RPC: Isoforma C del receptor de progesterona

RT-qPCR: Reacción en cadena de la polimerasa cuantitativa en tiempo real

7. List of abbreviations

A260: Absorbance at 260 nm
ABC: Avidin-Biotin-Peroxidase Complex
AI: Apoptotic index
BLAST: Basic Local Alignment Search Tool
CMT: Canine mammary tumors
Ct: Cycle threshold
CV: Coefficient of variation
DNA: Deoxyribonucleic acid
E: Efficiency
ER: Estrogen receptor
FFPE: Formalin-fixed, paraffin embedded
H&E: Haematoxylin and eosin
h: Hours
HPTR1: Hypoxanthine phosphoribosyl-transferase 1
IHC: Immunohistochemistry
M: Molar
mg: Milligrams
mL: Milliliters
mRNA: Messenger ribonucleic acid
P: Progesterone
PBS: Phosphate buffered saline
PI: Proliferation index
PR: Progesterone receptor
PRA: Progesterone receptor isoform A
PRB: Progesterone receptor isoform B
RPL32: Canine ribosomal protein L32
RPMI: Roswell Park Memorial Institute
RT-qPCR: Real time quantitative polymerase chain reaction
RU486: Mifepristone
RU534: Aglepristone
SD: Standard deviation
TUNEL: Terminal deoxynucleotidyl tranferase-mediated dUTP nick end labeling
ZK299: Onapristone

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8. Agradecimientos

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