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**PAPEL DEL TRANSPORTADOR ABCG2/BCRP Y SUS
POLIMORFISMOS EN LA SECRECIÓN ACTIVA DE
COMPUESTOS A LECHE Y SU MODULACIÓN
A TRAVÉS DE COMPUESTOS NATURALES EN
RUMIANTES**

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Artículo I: The bovine ATP-binding cassette transporter ABCG2 Tyr581Ser single nucleotide polymorphism increases milk secretion of the fluoroquinolone danofloxacin

Otero, J.A., Real, R., de la Fuente, A., Prieto, J.G., Marqués, M., Álvarez, A.I., Merino, G.

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Artículo II: The gain-of-function Y581S polymorphism of the ABCG2 transporter increases secretion into milk of danofloxacin at the therapeutic dose for mastitis treatment

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Artículo III: Effect of the bovine ABCG2 Y581S polymorphism on plasma pharmacokinetics and secretion into milk of enrofloxacin and its active metabolite ciprofloxacin

Otero, J.A., García-Mateos, D., de la Fuente, A., Prieto, J.G., Álvarez A.I., Merino, G.

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Artículo IV: Effect of bovine ABCG2 polymorphism Y581S SNP on secretion into milk of enterolactone, riboflavin and uric acid

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Artículo V: Role of ABCG2 in transport of the mammalian lignan enterolactone and its secretion into milk in Abcg2 knockout mice

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ABREVIATURAS

A-B: Apical-Basal

ABC: *ATP-Binding Cassette*/Casete de unión al ATP

ATP: Adenosin Trifosfato

AUC: Área bajo la curva

AUC (0-∞): Área bajo la curva de concentración del fármaco frente al tiempo desde el tiempo cero hasta el infinito

B-A: Basal-Apical

BCRP: *Breast Cancer Resistance Protein*

C_{máx}: concentración máxima

DMEM: *Dulbecco's Modified Eagle's Medium*/Medio Eagle modificado de Dubelcco

EMEA: *European Medicine Agency*/Agencia Europea del Medicamento

HPLC: *High pressure liquid chromatography*/Cromatografía líquida de alta resolución

Ko143: 3-(6-isobutil-9-metoxi-1,4-dioxo 1,2,3,4,6,7,12,12^a-octahidropirazino[1',2':1,6]pirido[3,4-b]indol-3-yl)-ácido propiónico éster ter-buril)

LC/MS: *Liquid chromatography/Mass spectrometry*/Cromatografía líquida/Espectrometría de masas

MCF-7: Línea celular procedente de células tumorales de mama

MDCK-II: *Madin-Darby Canine Kidney Cells*/Línea celular derivada de riñón de perro

MDR: *Multidrug Resistance*/Multirresistencia a fármacos

MF: Mediana de fluorescencia

MRP: *Multidrug Resistance Protein*/Proteína asociada a la resistencia de fármacos

MRT: Tiempo medio de residencia

MS: *Mass Spectrometer*/Espectrómetro de Masas

MXR Mitoxantrona

PAR: Parental

PBS: *Phosphate buffer saline*/Tampón fosfato salino

PCA: *Principal component analysis*/Análisis de componentes principales

PCR: *Polymerase Chain Reaction*/Reacción en cadena de la polimerasa

PDA: *Photodiode Array Detector*/Detector de formación de fotodiodos

P-gp: Glicoproteína P

PhIP: 2-amino-1-metil-6-fenilimidazol [4,5-b] piridina

qRT-PCR: *Quantitative Real Time Polymerase Chain Reaction*/Reacción en cadena de la polimerasa cuantitativa en tiempo real

QTL: *Quantitative trait locus*

SDG: Secoisolariciresinol diglucósido

SECO: Secoisolariciresinol

SNP: *Single Nucleotide Polymorphism*/Polimorfismo de nucleótido único

SP: *Side Population*/Población lateral

T_{1/2}: Vida media de eliminación

T_{máx}: Tiempo al que se alcanza la concentración máxima

1. RESUMEN

La proteína ABCG2/BCRP es un transportador de todo tipo de sustancias que se expresa en multitud de tejidos y órganos como el hígado, el intestino, la barrera hematoencefálica y la glándula mamaria (van Herwaarden and Schinkel, 2006). Este transportador afecta a la disponibilidad de sus sustratos e interviene en la secreción activa de xenobióticos y compuestos naturales a leche (van Herwaarden et al., 2007). La aparición de residuos de fármacos en leche es un problema de salud pública ya que puede desencadenar la aparición de alergias, resistencias bacterianas o hipersensibilidad (McManaman and Neville, 2003). Por otro lado, la transferencia de nutrientes específicos a leche puede ayudar a desarrollar productos de mayor calidad para los consumidores. Es por ello, que la identificación y el estudio de los factores involucrados en el transporte de fármacos y compuestos naturales a leche es una prioridad para el ámbito de la veterinaria y la industria láctea.

Se ha identificado un SNP en el ABCG2 bovino con un efecto importante sobre el QTL (quantitative trait locus) en el cromosoma 6 afectando a la composición y cantidad de la leche de vacas Holstein y roja noruega (Cohen-Zinder et al., 2005; Olsen et al., 2007). Una tarea de indudable interés es determinar si esta sustitución de aminoácido afecta al transporte de compuestos mediado por ABCG2 en ganado vacuno, pudiendo influir en los niveles sanguíneos y de leche tanto de nutrientes como de sustancias endógenas y fármacos, afectando por tanto a la disponibilidad de sus sustratos y a la calidad de la leche. Estudios iniciales de expresión transitoria en fibroblastos ovinos llevados a cabo por Merino et al., (2009) demostraron diferencias significativas en la acumulación de mitoxantrona entre las dos variantes del ABCG2 bovino. Posteriormente nuestro grupo de investigación demostró que la variante Y581S bovina mostraba una mayor actividad en cuanto al transporte *in vitro* de algunas fluoroquinolonas (Real et al., 2011b), y observamos además que ambas variantes pueden ser inhibidas de forma diferencial (Real et al., 2011b). Sin embargo, la relevancia de estos resultados *in vivo* permanecía sin explorar hasta el momento.

Estos estudios previos pusieron de relieve la potencial relevancia del transportador ABCG2, señalando de forma específica las posibles implicaciones para la industria láctea derivadas de la interacción de fármacos y compuestos naturales en la glándula mamaria con el transportador ABCG2 de rumiantes y en concreto el efecto del polimorfismo bovino Y581S de ABCG2. Por ello, la presente memoria tiene como objetivo principal la caracterización del efecto de este polimorfismo bovino *in vivo* así como la determinación de los efectos de dietas específicas sobre la secreción en leche de fármacos mediada por ABCG2 de rumiantes, para lo cual hemos realizado un conjunto de experimentos que se ven recogidos en cada uno de los artículos de esta memoria.

En nuestro **primer artículo** conseguimos poner a punto una técnica de genotipado bovino que nos permitió realizar un estudio farmacocinético con danofloxacino, un antimicrobiano ampliamente utilizado en veterinaria para tratar infecciones, en vacas Holstein portadoras y no portadoras del polimorfismo bovino Y581S a la dosis de 1,25 mg/kg. Este estudio demostró que en las vacas portadoras del polimorfismo se incrementaba la secreción de danofloxacino a leche con respecto a las vacas no portadoras ya que los ratios AUCs leche/plasma aumentaron de 4,09 a 8,81, corroborando los resultados que previamente se habían obtenido *in vitro* (Real et al., 2011b).

Estas diferencias en la secreción de danofloxacino a leche nos condujeron a un **segundo artículo**, en el que se testó una nueva formulación de danofloxacino a 6 mg/kg apropiada para el tratamiento de mastitis observándose también una diferencia en la secreción de fármaco a leche con un incremento de los ratios AUCs leche/plasma de 3,42 a 7,02. Asimismo, para determinar si las diferencias observadas en la secreción de fármaco a leche se debían a un incremento de la expresión de esta proteína en los individuos portadores del polimorfismo Y581S o un aumento de la actividad del transportador, se estudió la expresión proteica de ABCG2 bovino en función del polimorfismo a través de *western blot*. Los

resultados revelaron que en las células alveolares mamarias el polimorfismo bovino Y581S no afecta a la expresión de proteína por lo que teniendo en cuenta nuestros resultados, el polimorfismo Y581S podía ser considerado como un polimorfismo de ganancia de función *in vivo*.

En el **tercer artículo** estudiamos los efectos del polimorfismo Y581S sobre otro fármaco muy utilizado en la cabaña ganadera, el enrofloxacino y su metabolito activo ciprofloxacino. Los estudios farmacocinéticos sobre vacas Holstein portadoras y no portadoras del polimorfismo bovino Y581S demostraron que en las vacas portadoras la secreción de enrofloxacino a leche aumentaba significativamente con unos valores de los ratios AUCs leche/plasma de 0,52 en vacas Y/Y y 0,84 en vacas Y/S. Por otra parte la secreción de ciprofloxacino a leche también aumentaba en las vacas portadoras del polimorfismo con unos valores de los ratios AUCs leche/plasma de 3,00 en vacas Y/Y frente a 7,79 en vacas Y/S. Además se observó que el polimorfismo reducía la disponibilidad plasmática de ambos fármacos en torno a un 20%.

Dada la relevancia de ciertos nutrientes para la industria láctea en el **cuarto artículo** se estudió el efecto del polimorfismo sobre la secreción a leche de compuestos naturales con impacto sobre las propiedades nutricionales de la leche. Estos estudios revelaron que la enterolactona (enterolignano procedente de la dieta), la riboflavina (vitamina B₂) y el ácido úrico son sustratos del transportador ABCG2 bovino y son transportados de manera diferencial en función del polimorfismo Y581S *in vivo* y/o *in vitro*.

En el **quinto artículo** profundizamos en la interacción de la enterolactona con otras isoformas del transportador como son la murina y la humana demostrando que la enterolactona es un sustrato y un inhibidor *in vitro* de la isoforma humana y de ratón del transportador ABCG2. Los estudios *in vivo* con ratones *wild-type* y Abcg2^(-/-) también demostraron que ABCG2 es el principal responsable de la secreción de enterolactona a leche.

Dadas la propiedades nutritivas de la enterolactona y su capacidad para inhibir *in vitro* las isoformas bovina, murina y humana en el **sexto artículo** se estudió su actividad inhibitoria sobre la isoforma ovina concluyendo que la enterolactona es un eficiente inhibidor *in vitro* del transporte transepitelial de danofloxacino mediado por ABCG2 ovino, con ratios de transporte basal-apical/apical-basal de 9,22 en las células controles que sobreexpresan el transportador frente a ratios de 1,36 en presencia de enterolactona. Estos excelentes resultados *in vitro* dieron lugar a la realización de un estudio farmacocinético *in vivo* en ovejas de producción lechera de raza Assaf que mostró que las dietas enriquecidas con un 10 y 15 % en lino, como fuente natural de precursores de la enterolactona, y administradas durante 15 días, disminuían la secreción de danofloxacino a leche ya que los ratios correspondientes de AUCs leche/plasma se redujeron de 11,12 a 7,64 en los animales con la dieta.

Finalmente y como consecuencia del fuerte impacto del polimorfismo Y581S sobre la disponibilidad de sus sustratos en plasma y leche se decidió estudiar como un **anexo** a estos resultados el efecto del mismo sobre las propiedades fisicoquímicas de la leche. Asimismo también se realizó un estudio diferencial del metaboloma de estas vacas a nivel de plasma, leche y orina. Estos estudios concluyeron que el polimorfismo bovino Y581S de ABCG2 tiene efectos específicos sobre propiedades fisicoquímicas de la leche como la conductividad y la cantidad de lactosa. También se observaron efectos generales sobre la metabolómica de las vacas portadoras del polimorfismo, aumentando la concentración de ciertos metabolitos en leche y orina y reduciéndose en plasma.

Los resultados obtenidos en la presente memoria aportan información relevante acerca del papel de ABCG2 en la concentración de fármacos y compuestos naturales en la leche, así como la posibilidad de modular su actividad para obtener productos de mayor calidad para la industria y los consumidores.

2. INTRODUCCIÓN

Dentro del ámbito que abarca el sector ganadero y más concretamente dentro de la industria alimentaria se encuentra el sector lácteo que constituye un pilar básico para la economía local y nacional. Uno de los problemas que tiene este sector es la presencia potencial de residuos farmacológicos y de contaminantes medioambientales en la leche procedente de rumiantes. Estos residuos constituyen un riesgo para la Salud Pública ya que provocan la aparición de cepas resistentes y susceptibles de ser transmitidas al hombre, además de reacciones de hipersensibilidad, efectos tóxicos específicos o alteraciones de la flora intestinal, entre otros. Aparte del problema para el consumidor y el ganadero que pueden suponer los residuos de fármacos y tóxicos en la leche, la concentración en la leche de determinados compuestos endógenos o presentes en la dieta de rumiantes puede influir directamente en la seguridad y calidad del producto final. Por lo tanto, el estudio de los factores que afectan a la presencia de estos compuestos en la leche es crucial para la búsqueda de estrategias encaminadas a la obtención de leche de calidad, segura y saludable.

En los últimos años se ha demostrado que uno de los principales determinantes de la secreción activa de fármacos y compuestos endógenos a la leche es la proteína *Breast Cancer Resistance Protein* (BCRP/ABCG2), un miembro de la familia de los transportadores dependientes de ATP. Estas proteínas funcionan como bombas de expulsión de una gran variedad de compuestos no relacionados estructuralmente. Los transportadores ABC se expresan en diferentes barreras fisiológicas (tales como intestino, hígado y riñón) y limitan el paso de xenobióticos al organismo y la toxicidad tisular. De la misma manera, también se ha demostrado la presencia de ABCG2 en la glándula mamaria en lactación (Jonker et al., 2005) así como su papel esencial en la secreción activa a leche de sustratos de dicho transportador tales como antibióticos, agentes carcinógenos, toxinas y compuestos endógenos.

En humanos, se han descrito ampliamente diversos polimorfismos del transportador ABCG2 que dan lugar a diferente funcionalidad de la proteína y que son causantes directos de afecciones específicas como la gota (Woodward et al., 2009).

Una de las cuestiones que permanece sin explorar en animales de abasto es conocer si la existencia de variaciones genéticas o polimorfismos puede afectar a la actividad del transportador y ser la causa de variaciones en la secreción láctea de fármacos y otros sustratos, en aquellos animales que presenten dichos polimorfismos. En 2005 Cohen-Zinder et al., (2005) identificaron una mutación en el gen *ABCG2* con un efecto en la composición y cantidad de la leche de vacas Holstein y roja noruega. Este SNP (A→C) codifica la sustitución de una tirosina por una serina (Y581S). Se ha sugerido que este polimorfismo del transportador *ABCG2* puede tener un papel importante por su potencial implicación en las diferencias en el transporte de fármacos y compuestos naturales a leche.

Gracias al modelo *in vitro* usando células MDCK-II puesto a punto previamente por nuestro grupo, se ha demostrado recientemente la capacidad diferencial de ambas variantes genéticas del transportador para transportar *in vitro* sustratos con relevancia en la medicina veterinaria, mostrando la variante Y581S una mayor capacidad de transporte (Real et al., 2011b). Sin embargo, hasta el momento no existían estudios *in vivo* que corroboraran la validez y solidez de estos resultados obtenidos en el laboratorio. Es por ello que esta tesis doctoral se centrará en el estudio *in vivo* de la implicación del polimorfismo bovino Y581S de *ABCG2* en la biodisponibilidad y secreción de fármacos utilizados en ganadería, así como en el efecto de este polimorfismo en la composición de la leche a nivel metabólico, fisicoquímico y nutricional. Además, con el objeto de mejorar las propiedades nutricionales de la leche y al mismo tiempo de reducir la eventual secreción de fármacos a leche se evaluará el efecto de dietas específicas en la modulación de la actividad de *ABCG2* en ovejas.

3. REVISIÓN BIBLIOGRÁFICA

3.1. Características generales de los transportadores ABC (ATP Binding Cassette)

Los transportadores son proteínas especializadas que se encuentran embebidas en membranas lipídicas y que intervienen en la translocación de compuestos químicos de distinta naturaleza dentro y fuera de la célula tanto de manera activa como pasiva. Se denomina transporte pasivo cuando una molécula atraviesa una membrana lipídica a través de una proteína a favor de gradiente de concentración. Se considera transporte activo cuando determinadas moléculas se transportan a través de membranas biológicas en contra de un gradiente de concentración consumiendo energía. Si el proceso de transporte activo se acopla al transporte de otro sustrato (típicamente un ion) para obtener la energía, se denomina cotransporte. En función del sentido del transporte de estos sustratos se considera un proceso de simporte o antiporte. Si ambos compuestos químicos se transportan en el mismo sentido el proceso se denomina simporte y si son transportados en direcciones opuestas se considera antiporte (Sadée et al., 1995) (Figura 1). Por otro lado, existen proteínas que obtienen la energía necesaria para transportar sus sustratos activamente de la hidrólisis de ATP. Entre estas proteínas se encuentran los transportadores de membrana dependientes de ATP, también conocidos como transportadores ABC (ATP Binding Cassette) y que actúan como transportadores activos primarios o “bombas exportadoras” (Liang et al., 2015).

Figura 1. Tipos de transporte activo mediado por proteínas (Purves et al., 1994).

Los transportadores ABC están ampliamente distribuidos en todos los seres eucariotas, lo cual indica su relevancia para multitud de funciones celulares y fisiológicas. La elevada conservación de los genes que codifican para los transportadores ABC es un claro indicador de la importancia de estas proteínas para el desarrollo de funciones biológicas básicas. Algunas de estas funciones incluyen el transporte de lípidos, sales biliares, colesterol, hormonas, esteroides, metabolitos y el de otros compuestos considerados tóxicos (Vlaming et al., 2009). La estructura básica que define a los miembros de esta familia de proteínas es la combinación de un lugar de unión al ATP y diferentes segmentos transmembrana. En mamíferos, las proteínas ABC funcionalmente activas contienen típicamente doce segmentos transmembrana distribuidos en dos mitades homólogas, dos zonas de unión al ATP ubicadas en la parte citoplasmática y un sitio de glicosilación. Esta estructura básica puede darse en una única cadena polipeptídica (*full-transporters*), o en dos proteínas separadas (*half-transporters*) (Figura 2). En este último caso el transportador ABC pasaría a ser activo después de una dimerización específica (Robey et al., 2009).

Figura 2. a) Esquema representativo de la estructura de los *full-transporters* ABCB1 y ABCB11 b) Esquema representativo de la estructura de los *full-transporters* de cadena larga ABCC1, 2 y 3 c) Disposición en la membrana plasmática de un *half-transporter* ABC previa a su dimerización. (Ferreira et al., 2014).

Algunos de los transportadores ABC son altamente específicos, y reconocen únicamente un número limitado de sustratos; otros sin embargo presentan una baja especificidad y son capaces de reconocer una amplísima variedad de compuestos estructuralmente diferentes, incluyendo toxinas y metabolitos presentes en la dieta, agentes carcinogénicos y diversos fármacos (Wong et al., 2014). La función fisiológica que se atribuye a estos transportadores es la de protección del organismo frente a compuestos tóxicos y la del transporte de

sustancias endógenas a otros compartimentos para que cumplan una determinada función fisiológica. Todos ellos se localizan en las membranas lipídicas de orgánulos o en zonas basolaterales y/o apicales de la membrana plasmática. La secuenciación del genoma humano ha permitido caracterizar, hasta el momento, 49 genes ABC que codifican para 44 transportadores ABC (Barbet et al., 2012), 14 de los cuales están asociados con enfermedades genéticas (Vasiliou et al., 2009). Los que han sido estudiados en mayor profundidad son la glicoproteína P (P-gp/ABCB1), las proteínas asociadas a la resistencia de fármacos (MRPs 1-2/ABCCs 1-2) y *Breast Cancer Resistance Protein* (BCRP/ABCG2). Dado el amplio espectro de sustancias que son capaces de reconocer, algunos de estos transportadores comparten simultáneamente el transporte de uno o varios sustratos (Estudiante et al., 2013). Aunque parezca sorprendente, la naturaleza desarrolla en elevado número de ocasiones mecanismos redundantes que apuntalan el adecuado funcionamiento de actividades biológicas vitales. Es por ello que como estudiaremos más adelante, en aquellos tejidos donde se expresan diferentes transportadores, la deficiencia de uno de ellos se puede ver compensada por la actividad de otro transportador (de Waart et al., 2009; Vlaming et al., 2011; Durmus et al., 2015).

La capacidad que tienen los transportadores de ser inducidos bajo una determinada presión biológica hace que en un momento determinado estos mecanismos puedan descontrolarse y desencadenar y/o promover el desarrollo de enfermedades. De hecho, otra de sus principales características que ha motivado el descubrimiento y el estudio en profundidad de estas proteínas es su estrecha relación con el fenómeno de la multirresistencia a fármacos antitumorales (Choi and Yu, 2014). Dado que estas proteínas poseen la capacidad de reconocer y transportar toxinas y compuestos potencialmente tóxicos para las células, en algunos tumores se produce una sobreexpresión de estos transportadores que disminuye la entrada de fármacos antitumorales en las células, reduciendo la eficacia de

estos tratamientos y favoreciendo la supervivencia de células tumorales. Desgraciadamente, esta inducción suele tener un carácter inespecífico en cuanto al grado de sobreexpresión y en cuanto a los transportadores que se ven involucrados (Moreira et al., 2014). No se conocen detalladamente los procesos que conducen a este fenómeno por lo que aún no existen terapias personalizadas que aborden este proceso de inducción en el desarrollo de la enfermedad que permitirían mejorar el pronóstico de las patologías afectadas por el fenómeno la multirresistencia a fármacos (Kathawala et al., 2015). Entre todos los transportadores pertenecientes a la familia ABC, hay 13 que se relacionan con el transporte y la resistencia a fármacos (Gillet et al., 2007). A continuación vamos a profundizar en los más importantes desde el punto de vista farmacológico.

3.2. Glicoproteína P /ABCB1

La proteína P-gp (ABCB1) es un polipéptido de 170 KDa en humanos organizado en tándem, es decir, en un conjunto de dos elementos que se complementan, constituidos por aminoácidos repetidos y unidos a través de una región específica. Esta proteína es el transportador ABC más estudiado y mejor conocido hasta el momento debido al elevado número de trabajos que han investigado su funcionamiento y estructura. Se descubrió inicialmente porque se expresaba en células derivadas de ovario de hámster chino que eran resistentes a colchicina (Juliano and Ling, 1976). Se expresa en mayor grado en la membrana apical de los enterocitos, en las células del canalículo biliar y en las células del túbulo proximal del riñón. Dado que todas estas células pertenecen a órganos con capacidad secretora, se cree que la función principal de esta proteína consiste en la eliminación de sustancias endógenas y exógenas del organismo (Silva et al., 2015). De hecho, también se ha detectado una elevada expresión de ABCB1 en las barreras hematoencefálica, hematotesticular y fetoplacentaria, lo que refuerza el carácter protector de esta proteína. Sin embargo, ABCB1 también se expresa en otros tipos celulares muy diferentes a los mencionados para los cuales

su función no es tan obvia tal y como sucede con las células hematopoyéticas, por lo que hay procesos derivados de la actividad de este transportador que aún están por describir (Eckford and Sharom, 2009). Entre los sustratos de ABCB1 encontramos multitud de fármacos de uso generalizado y ampliamente utilizados en medicina humana como la ciclosporina A, la digoxina, la morfina, así como una amplio número de antitumorales (Silva et al., 2015).

Dentro de un tumor, también existen poblaciones de células troncales tumorales que han mostrado una elevada expresión del transportador ABCB1. Tanto es así que estas células se pueden seleccionar dentro de una población celular compleja gracias a su capacidad para transportar el sustrato de ABCB1 fluorescente Hoechst 33342 (Shi et al., 2008). No se conoce qué papel juega este transportador en estas células troncales tumorales aunque las evidencias apuntan a que confiere a estas células la capacidad sobrevivir a terapias contra el cáncer. Dada su capacidad para multiplicarse de manera indefinida, la supervivencia de estas células mediada por la sobreexpresión de este transportador permitiría la reaparición del tumor tras un tratamiento quimioterápico como se ha visto para el hepatocarcinoma (Shi et al., 2008).

En cualquier caso, la función protectora de ABCB1 parece ser la principal función de esta proteína, ya que aunque la generación de ratones *Abcb1* a/b ^(-/-) en esta proteína no parece comprometer la viabilidad y supervivencia de los mismos, hay un claro aumento de la susceptibilidad a xenobióticos. El ejemplo más ilustrativo a este respecto es el del antiparasitario ivermectina para el cual los ratones *Abcb1* a/b ^(-/-) pasan a tener una susceptibilidad 100 veces mayor que los ratones *wild-type*, presentando neurotoxicidad aguda en los animales que carecen de esta proteína (Schinkel et al., 1994). Estos hallazgos fueron corroborados posteriormente por estudios *in vitro* que demostraron que el transportador ABCB1 es capaz de reconocer y transportar este antiparasitario (Schinkel et al., 1996).

Dada la relevancia fisiológica de este transportador, sus variantes genéticas tienen efectos notables sobre la interacción con determinados fármacos. El *National Center for Biotechnology Information* (NCBI) almacena información sobre 50 variantes distintas de este gen en humana, entre las cuales destaca la mutación 3435C>T (rs1045642) que reduce la expresión intestinal del transportador y altera la absorción de fármacos sustratos (Manna et al., 2015).

3.3. MRP1/ABCC1

MRP1 es una proteína de 190 KDa en humanos codificada por el gen *ABCC1* que juega un papel importante en la disposición de agentes citotóxicos en células normales (Cole, 2014). De todos los transportadores de la subfamilia C, *ABCC1* es el transportador cuya distribución en el organismo es más amplia; se considera prácticamente ubicuo en todos los tejidos, aunque su expresión es más relevante en aquellos tejidos que actúan protegiendo órganos de vital importancia en la función corporal como son el cerebro (en la barrera hematoencefalica), las gónadas (en la barrera hematotesticular) y en el desarrollo del embrión (placenta). Al contrario que *ABCB1*, el transportador *ABCC1* es un transportador que se expresa en la membrana basal de las células (Choi and Yu, 2014).

Una de las características más particulares de este transportador radica en su importante contribución en el control del estrés oxidativo celular (Cole, 2014). El glutatión es un tripéptido antioxidante crucial para proteger a las células de los efectos del estrés oxidativo. Cuando determinadas moléculas se conjugan con la forma reducida del glutatión, el transportador es capaz de reconocer estas sustancias y proceder al transporte de éstas fuera de la célula. Ambas formas, tanto la reducida (GSH) como la oxidada (GSSG) del glutatión se han descrito como sustratos. Sin embargo, el glutatión en su forma oxidada es transportado con mayor afinidad que la forma reducida, probablemente debido a que en su forma oxidada puede

actuar como un agente oxidante y por lo tanto ser potencialmente tóxico. De esta manera, ABCC1 contribuiría a una adecuada homeostasis del estrés oxidativo en la célula (Krause et al., 2007). Por otra parte, el glutatión reducido parece tener también la capacidad de cotransportar o estimular el transporte de determinadas moléculas sin tener que conjugarse con ellas necesariamente. Cómo la presencia de GSH puede promover el transporte de determinadas sustancias está aún por dilucidar de forma completa. Actualmente, se cree que el glutatión en su forma reducida puede inducir cambios en el plegamiento proteico que facilitaría el reconocimiento de sustancias estructuralmente tan diferentes como la dehidroepiandrostenediona o la estrona-3-sulfato que solo son transportadas en presencia del tripéptido (Cole, 2014).

En cualquier caso, este transportador es uno de los más estudiados por lo que el número de sustratos descritos hasta el momento es muy elevado y comprende desde compuestos endógenos como el derivado hormonal β -estradiol 17-(β -D-glucurónido) o el derivado enzimático bilirrubina-glucurónido hasta fármacos de diferente naturaleza como la grepafloxacina o el metotrexato (Klaassen and Aleksunes, 2010). Por todo ello, este transportador es uno de los principales responsables del desarrollo del fenómeno de multirresistencia a fármacos durante la formación de tumores; de hecho, varios estudios han correlacionado inversamente el éxito de las terapias antitumorales con la expresión de este transportador (Kathawala et al., 2015).

Entre los polimorfismos de ABCC1 destaca el G2012T por las complicaciones que produce en la absorción, distribución y eliminación de agentes terapéuticos afectando en última instancia a la eficacia de los tratamientos. Concretamente, los pacientes con cáncer portadores del polimorfismo G2012T tratados con antraciclinas como la doxorubicina presentan con frecuencia cuadros de cardiotoxicidad aguda sin embargo, en aquellos pacientes que presentan la variante *wild type*, los lisosomas de los cardiomiocitos secuestran

la doxorubicina a través de este transportador impidiendo efectos tóxicos a nivel de miocardio (Wojnowski et al., 2005).

3.4. MRP2/ABCC2

MRP2/ABCC2 es una proteína codificada por el gen *ABCC2* con un peso de 200 KDa. A pesar de su proximidad filogenética comparte con *ABCC1* menos de un 50% de la secuencia aminoacídica. Además, mientras que *ABCC1* se expresa principalmente en la membrana basolateral de células polarizadas, *ABCC2* se expresa en la membrana apical de las células. Se encuentra principalmente en órganos como el hígado, el riñón, el intestino y la vesícula biliar (Vlaming et al., 2011). *ABCC2* es especialmente eficiente en lo que respecta al transporte de aniones orgánicos conjugados con sulfato, ácido glucurónico y glutatión. Una de las funciones más significativas de este transportador consiste en la secreción biliar de sustancias (Klaassen and Aleksunes, 2010). Dada la importancia de esta proteína en la interacción y secreción de compuestos conjugados de fármacos, es uno de los transportadores más estudiados a la hora de hacer un seguimiento en el proceso de ADME (administración, distribución, metabolismo y excreción) por parte de las compañías farmacéuticas (de Waart et al., 2009). Entre los sustratos tipo para esta proteína encontramos el agente quimioterapéutico metotrexato y el cisplatino. Merecen una especial mención los fármacos de uso común como el probenecid, el MK 571, la ciclosporina A y la azitromicina, que ejercen un efecto inhibitorio sobre la actividad de la proteína y pueden dar lugar a interacciones farmacológicas (Chen and Tiwari, 2011).

Asociado a este transportador encontramos el síndrome de Dubin-Johnson en humanos que se caracteriza por una incapacidad para secretar bilirrubina conjugada, lo que lleva a una hiperbilirrubinemia que produce ictericia. Actualmente se han descrito varios polimorfismos en

el gen que codifica para esta proteína que afectan al funcionamiento de ABCC2 y que explican la aparición de la enfermedad como el R768W y W709R (Okada et al., 2014).

3.5. ABCG2/BCRP

ABCG2 es una de las 5 proteínas de la subfamilia G que incluye también a ABCG1, ABCG4, ABCG5, ABCG8. Todos ellos son *half-transporters* que forman homodímeros o heterodímeros. Algunos autores han hipotetizado con que varios dímeros se puedan asociar formando oligómeros superiores (Mo and Zhang, 2012). Mientras que en el resto de transportadores ABC, el dominio de unión a ATP se encuentra en el extremo C-terminal, en los transportadores ABCG este dominio está en el extremo N-terminal de la proteína. La actividad de los transportadores ABCG esta principalmente asociada al transporte de lípidos (Moitra et al., 2011), aunque ABCG2 es el que muestra un espectro de sustratos más amplio incluyendo compuestos tanto exógenos, como naturales de carácter hidrofóbico, aniónico, catiónico y metabolitos conjugados de fase II (Jani and Krajcsi, 2014)..

ABCG2/BCRP es un *half-transporter* que dimerizado tiene un peso de 72 KDa en humanos. La denominación BCRP (*Breast Cancer Resistance Protein* ó Proteína de Resistencia al Cáncer de Mama) fue dada cuando este transportador fue descubierto en 1998 al ser aislado de una línea celular de cáncer de mama multirresistente a la mitoxantrona y al topotecan entre otros (Doyle et al., 1998). La lista de sustratos e inhibidores xenobióticos ha crecido exponencialmente desde entonces. De manera general, ABCG2 se expresa en la membrana apical de las células en órganos que contribuyen a los procesos de ADME. Por tanto, ABCG2 es un transportador muy versátil con multitud de funciones y su papel en distintas ramas de la fisiología, farmacología y toxicología le convierte en un transportador de amplio interés para la comunidad científica y médica (Jani and Krajcsi, 2014).

3.5.1. Estructura de la proteína

A diferencia de otras proteínas ABC, para ABCG2 aún no ha sido posible la determinación de la ultraestructura proteica de alta resolución. Hasta ahora solo ha sido posible el análisis de la proteína en 3D a baja resolución y la cristalización bidimensional con la que se demostró que la presencia de determinados sustratos como la mitoxantrona inducían cambios conformacionales en ABCG2 (Rosenberg et al., 2010) y que los sitios de unión al ATP se encontraban bastante separados entre sí (Rosenberg et al., 2015). Actualmente se sabe que ABCG2 es un *half-transporter* que consta de un dominio de unión a nucleótidos (residuos 1 a 396) y un dominio transmembrana que a su vez está compuesto por 6 α -hélices (residuos 397 a 655). Ambos extremos N- y C- terminal están localizados en el citoplasma (Figura 2) (Jani and Krajcsi, 2014). De los 3 posibles lugares de glicosilación, sólo una asparragina en posición 596 del lazo extracelular parece estar glicosilada, pero ésta no resulta imprescindible para la correcta localización y funcionalidad del transportador (Diop and Hrycyna, 2005). Tras años de controversia, finalmente los estudios de Haider et al., (2011) corroboraron que los homómeros, dimerizan para dar lugar a un homodímero o heterodímero funcional y que se unen a través de enlaces disulfuro. Recientes estudios han relacionado a otras proteínas con el proceso de dimerización por lo que se cree que podrían actuar regulando la formación y por tanto la actividad de ABCG2 (Zhou et al., 2013).

Por otra parte, los estudios cinéticos sobre esta proteína revelan que este transportador consta de diferentes sitios de unión a sustrato como se ha demostrado con los sustratos rodamina y Hoechst 33342, lo cual afecta tanto al transporte de sustancias como a la inhibición (Clark et al., 2006). Los estudios apuntan a que ABCG2 consta de dos sitios de unión a sustrato (Glavinas et al., 2007). A pesar de toda la información que existe al respecto aún no está claro el mecanismo a través del cual los sustratos acceden al transportador.

Aunque inicialmente se creía que los sustratos entraban directamente al transportador desde el citoplasma (Aleksunes et al., 2008), los estudios más recientes han demostrado que, al menos determinados sustratos, necesitan insertarse en la membrana antes de llegar al transportador y ser exportados fuera de la célula (Sharom, 2008).

3.5.2. Distribución tisular y función fisiológica

La expresión de ABCG2 se extiende a la práctica totalidad de los tejidos y tipos celulares, aunque el grado de expresión puede ser muy diferente. Entre todos ellos el que alcanza un mayor grado de expresión es el **sincitiotrofoblasto** de la placenta protegiendo al feto de la entrada de sustancias potencialmente dañinas para el desarrollo embrionario.

En **hígado**, ABCG2 se expresa en la membrana canalicular de los hepatocitos (Figura 3), en los conductos y conductillos biliares, los vasos sanguíneos del endotelio hepático y en la membrana luminal de las células epiteliales de la vesícula biliar (Vander Borgh et al., 2006). ABCG2 interviene en el transporte de diversos compuestos hacia el canalículo biliar y por tanto, en su excreción a bilis (Merino et al., 2005a).

Figura 3. Órganos en los que se localiza el transportador ABCG2. Las flechas pequeñas indican la dirección del transporte mediado por ABCG2 en cada órgano. Las flechas grandes indican la excreción neta en el organismo de los sustratos de ABCG2. La expresión en células endoteliales, capilares y venas en los distintos tejidos no aparece indicada (Real et al., 2011b).

En **riñón**, la proteína se localiza en el túbulo cortical y en el borde en cepillo de las células del túbulo proximal, participando en la excreción de fármacos y compuestos endógenos como el ácido úrico (Woodward et al., 2009) aunque el nivel de expresión que alcanza en este órgano en el caso de los humanos es relativamente bajo en comparación con el del intestino (Mao and Unadkat, 2015).

ABCG2 se expresa en la membrana apical del **intestino delgado y grueso**, evitando la entrada de sustancias potencialmente dañinas para el organismo. Se ha demostrado que el transportador limita la absorción de compuestos antitumorales como el topotecan, la mitoxantrona e inhibidores de la tirosina kinasa (TKI) entre otros. También limita la penetración de sustancias no terapéuticas como metabolitos de fase II (principalmente

sulfatados) así como porfirinas y derivados hormonales como la estrona-3-sulfato. Se ha demostrado que el uso de inhibidores puede frenar la actividad del transportador e incrementar la biodisponibilidad de sus sustratos (Estudante et al., 2013). Por otro lado, se ha observado que los tratamientos crónicos con sustratos de ABCG2 tienen la capacidad de inducir la expresión del transportador en el intestino (Peroni et al., 2011) potenciando el efecto ejercido de este transportador sobre otros compuestos. Tanto ABCB1 como ABCC1 colaboran con ABCG2 para expulsar compuestos al lumen intestinal. Así, el uso del inhibidor de ABCG2 GF120918, que también inhibe al transportador ABCB1, aumenta en más del doble la biodisponibilidad del topotecan (Estudante et al., 2013). Además, los polimorfismos de ABCG2 a nivel intestinal pueden alterar drásticamente la absorción y los parámetros farmacocinéticos de determinados sustratos como la 9-aminocamptotecina, el diflomotecan, el irinotecan, la rosuvastatina, la sulfasalazina y el topotecan (Estudante et al., 2013).

Dado que una de las principales funciones fisiológicas de este transportador es limitar la penetración sistémica de sus sustratos (Figura 3) el transportador se expresa en determinadas regiones del **endotelio capilar** de los vasos sanguíneos devolviendo a sus sustratos al torrente sanguíneo como sucede en la barrera hematoencefálica, de manera que se protege al cerebro de la entrada de compuestos potencialmente tóxicos (Mao and Unadkat, 2015).

En 2005 se añadió la **glándula mamaria** en lactación a la lista de tejidos que expresaban una elevada cantidad del transportador (Jonker et al., 2005). Concretamente se expresa en el dominio apical del epitelio alveolar mamario (Figura 4) y la expresión del transportador se ve fuertemente inducida en humanos, ratón y vaca durante la lactación.

Figura 4. Detección inmonohistoquímica de **ABCG2** en C) Ratón hembra virgen, D) Ratón hembra en lactación (una semana), E) Vaca virgen, F) Vaca en lactación, G) Mujer, y H) Mujer en lactación (Jonker et al., 2005).

La función de este transportador en este tejido agrega una interesante incógnita al rol protector que inicialmente se le achacó al transportador. La localización apical de este transportador en glándula mamaria conlleva la concentración de sus sustratos (entre los que se encuentran compuestos tóxicos) en la leche comprometiendo potencialmente la supervivencia de la descendencia (Jani and Krajcsi, 2014). Este mecanismo ha sido ampliamente estudiado en lo que se refiere a la acumulación de toxinas en leche mediada por ABCG2 (Real et al., 2011a). Por otra parte, entre los sustratos de ABCG2 también se encuentran vitaminas como la riboflavina, el ácido fólico y la vitamina K₃, lo cual explicaría la relevancia de este transportador para la producción láctea. Sin embargo, estudios con ratones *Abcg2*^{-/-} demuestran que la cantidad de vitaminas en leche no se ve afectada por la ausencia de este transportador y ni tampoco se detecta una deficiencia de las mismas en las crías, al menos en un entorno de laboratorio. Por tanto, aunque ABCG2 parece transportar vitaminas,

la relevancia fisiológica de este proceso en la glándula mamaria queda en entredicho probablemente debido a la existencia de otro mecanismo compensatorio de transporte de vitaminas que eclipsaría el efecto de ABCG2 (Jani and Krajcsi, 2014).

Recientemente se ha demostrado que el transportador Oct-4 también se expresa en glándula mamaria durante la lactación de manera que contribuye de manera activa al proceso de concentración de compuestos tóxicos y vitaminas a leche (Ito et al., 2014). La verdadera función biológica de estos transportadores en la glándula mamaria no se ha descifrado hasta el momento. Hay que tener en cuenta que otros transportadores ABC tales como ABCC2 o ABCB1 no presentan esa inducción de su expresión en glándula mamaria durante la lactación. Vlaming y cols. (2009) revisaron 5 hipótesis que pueden explicar la presencia y la función de la expresión de ABCG2 en la glándula mamaria.

- 1) La primera de ellas asume que la expresión de ABCG2 en la glándula mamaria es casualidad lo cual implicaría que carece de una función biológica esencial. Esta hipótesis explicaría por qué no es posible detectar un fenotipo en las crías de los ratones *Abcg2*^(-/-). A pesar del efecto negativo de la concentración de compuestos tóxicos en la leche, la cantidad de toxina que llega a la descendencia es muy pequeña ya que en realidad la progenitora elimina gran parte de las toxinas por otros mecanismos. La secreción de vitaminas puede ser un efecto derivado de la diversidad de sustratos que reconoce el transportador. Sin embargo, el hecho de que el transportador sea específicamente inducido en la lactación en todas aquellas especies en las que se ha estudiado hasta al momento parece indicar que existe alguna presión evolutiva por parte del algún factor desconocido para que esta inducción se siga dando.
- 2) La segunda hipótesis explica que la madre utiliza la secreción mamaria como un mecanismo más para aclarar las toxinas que llegan a su organismo. Posteriormente el lactante se libraría de ellas a través de sus propios mecanismos. Aunque esta

hipótesis ha sido apoyada por otros autores (Lindner et al., 2013), deja muchas incógnitas sin resolver ya que en individuos lactantes los sistemas de eliminación de compuestos tóxicos no están plenamente desarrollados e incluso bajas concentraciones pueden ser muy dañinas para las crías. A este respecto tendría un mayor sentido biológico promover la inducción de ABCG2 en otros órganos como en el hígado para hacer más eficiente la eliminación de sustancias por otras rutas sin comprometer la supervivencia de las crías.

- 3) La tercera hipótesis defiende que la expresión de ABCG2 es un mecanismo de preparación de los lactantes para el cambio a los alimentos sólidos. Dado que es probable que las crías terminen ingiriendo los mismos alimentos que la madre y por tanto sean expuestos a los mismos tóxicos, la madre va sometiendo gradualmente a las crías a dichas sustancias para que el organismo se vaya adaptando. Sin embargo, aún no existen resultados concluyentes que demuestren que los sustratos de ABCG2 estimulan la maduración de las vías de detoxificación de las crías.
- 4) La cuarta hipótesis aboga por que la transferencia de xenobioticos a la leche previene el desarrollo de alergias en la edad adulta. Recientes estudios demuestran que el desarrollo de alergias se reduce si el recién nacido se expone a ciertos tóxicos de manera temprana. Aunque la mayoría de alérgenos son proteínas o proteínas conjugadas, también existen moléculas pequeñas que pueden desencadenar alergias. Aun es necesario describir alérgenos sustratos de ABCG2 para demostrar esta hipótesis.
- 5) La quinta hipótesis defiende que ABCG2 es necesario para la concentración de nutrientes esenciales a la leche. Este hecho está sujeto a la necesidad de encontrar unas condiciones de alojamiento de ratones *Abcg2*^(-/) en el animalario que hiciera visible un fenotipo, o que se encuentre un nuevo sustrato nutriente cuya deficiencia sí muestre un fenotipo en las crías. Sin embargo, esta hipótesis tiene el defecto de que

en ese caso sería evolutivamente más lógico la inducción de transportadores específicos para este nutriente en lugar de un transportador multiespecífico como ABCG2.

3.5.3. Importancia de los polimorfismos del transportador

La variabilidad genética es un factor que promueve el desarrollo evolutivo de los seres vivos a través de determinadas mutaciones genéticas que pueden provocar una alteración en la síntesis proteica y que en un momento determinado puede suponer una ventaja frente a un ambiente. Cuando estas mutaciones se fijan en una especie en una frecuencia >1% pasan a denominarse polimorfismos. Existen varios tipos de polimorfismos. Cuando el cambio se da en una única base nitrogenada estamos hablando de un polimorfismo de nucleótido simple o de un “single nucleotide polymorphism” (SNP). Si el SNP produce un cambio en la secuencia proteica que sintetiza el gen portador del polimorfismo se considera SNP no sinónimo, mientras que si no afecta a la secuencia aminoacídica se considera un SNP sinónimo. Existen multitud de polimorfismos asociados al transportador ABCG2 humano como por ejemplo: los polimorfismos no sinónimos 34G>A, 151G>T, 376C>T, 421C>A, 458C>T, 496C>G, 616A>C, 623T>C, 742T>C, 1000G>T, 1291T>C, 1465T>C, 1768A>T, y 1858G>A (Figura 5) (Noguchi et al., 2014).

Figura 5. Diagrama esquemático del transportador ABCG2 humano y sus SNPs. Las estrellas representan puntos con polimorfismos (Noguchi et al.,2014)

Los SNPs no sinónimos en los transportadores ABC producen cambios en la estructura primaria de la proteína lo cual suele conllevar una alteración del plegamiento de la proteína (más o menos acusada) y en consecuencia una alteración en la función de transporte. Como consecuencia de ello, se puede dar la circunstancia de que los polimorfismos de ABCG2 puedan en última instancia afectar la respuesta farmacológica de pacientes portadores de variantes genéticas distintas del transportador. En la población humana japonesa se han detectado tres variantes de este tipo en el transportador ABGC2. Los polimorfismos 34G>A (V12M), 421C>A (Q141K) y una delección de seis nucleótidos entre la posición 944 y 949 lo que conlleva la eliminación de los aminoácidos A315 y T316 (Mao and Unadkat, 2015). De todos ellos el más común es el SNP (Q141K). La frecuencia alélica en la población asiática es de entre el 27% y el 34%. En el resto de poblaciones la frecuencia de este polimorfismo

apenas llega al 10% (Mao and Unadkat, 2015). Este polimorfismo afecta al proceso de dimerización del transportador necesario para su funcionalidad. En consecuencia, los individuos portadores de este polimorfismo presentan una baja expresión de proteína funcional. Este polimorfismo tiene fuertes implicaciones farmacológicas y fisiológicas. Dado que el ácido úrico es un sustrato endógeno del transportador, los pacientes portadores de esta variante, presentan dificultades para excretar el ácido úrico a orina lo cual produce hiperuricemia y termina promoviendo el desarrollo de gota (Woodward et al., 2009). Asimismo, también presenta efectos frente a otros sustratos de ABCG2 que se administran de manera exógena como ocurre con ciertos fármacos. Los estudios en fase I con el diflomotecan demostraron que los individuos heterocigotos para este polimorfismo presentaban niveles plasmáticos tres veces más altos de este agente antitumoral que los no portadores (Sparreboom et al., 2004). Asimismo, los individuos portadores de este polimorfismo también presentaban una mayor biodisponibilidad de topotecan y de la 9-aminocamptotecina (metabolito activo del antiqumioterapéutico camptotecina) (Noguchi et al., 2014). También se ha asociado a este polimorfismo con un incremento en los efectos diarreicos producidos por el tratamiento con gefitinib (Zamboni et al., 2006).

Además de estos, existen otros polimorfismos no sinónimos que afectan al nivel de expresión de proteína en la membrana plasmática ya que parecen desestabilizar la proteína, promoviendo una mayor proteólisis del transportador mediada por ubiquitina, lo cual reduce la expresión del transportador (Noguchi et al., 2014). También con relevancia fisiológica, aunque menos prevalente en la población japonesa y casi inexistente en la caucásica, encontramos el SNP 376C>T (Q126stop) que provoca una finalización prematura de la traducción de esta proteína (Noguchi et al., 2014).

Por último también se han descrito para ABCG2 humano un gran número de polimorfismos genéticos sinónimos como 114T>C, 369C>T, 474C>T, 564A>G, 1098G>A, y

1425A>G. Se cree que algunos de estos polimorfismos pueden influir en el grado de expresión de proteína aunque los trabajos dedicados al estudio de este tipo de polimorfismos son muy limitados por el momento y aun no se conocen en profundidad sus efectos (Noguchi et al., 2014).

3.6. Implicaciones de ABCG2 en el ámbito veterinario

Toda la información y el conocimiento desarrollado en torno al transportador ABCG2, a través de los estudios en células que lo sobreexpresan de manera estable, los resultados obtenidos estudiando ratones *Abcg2*^(-/-) y los resultados derivados de los estudios clínicos en humana, indican que los resultados de la investigación sobre el transportador ABCG2 de forma general pueden hacerse extensivos a otros campos con importantes implicaciones en la salud pública, en la nutrición y en la producción animal, entre otros. Dada la relevancia e impacto de los estudios previamente realizados en el campo de la veterinaria desde el punto de vista farmacológico o clínico, el transportador ABCG2 presenta un gran potencial en estos ámbitos de investigación que puede ser explotado.

Como consecuencia de la amplitud de sustratos que reconoce este transportador, los efectos más trascendentes derivados de su estudio en este campo, engloban principalmente a la biodisponibilidad de fármacos y su secreción a leche que pueden verse afectados en animales por cambios endógenos (polimorfismos) o exógenos (interacciones sustrato-sustrato o sustrato-inhibidor) del transportador.

A pesar de que el modelo murino y el humano, proporcionan una sólida base para el estudio de la interacción de ABCG2 con sus sustratos, se hace necesario estudiar dicha interacción de forma directa en las especies de interés veterinario debido a los factores que se comentan a continuación.

El primer aspecto a considerar es que entre las diferentes especies los niveles de expresión de los transportadores pueden variar, lo que podría explicar parcialmente el porqué

de que ciertos parámetros farmacocinéticos sean tan diferentes entre especies, incluso para aquellas especies que comparten una alta homología genética. En especies filogenéticamente muy próximas como son la rata y el ratón se han visto grandes diferencias en la expresión de ABCG2 en un órgano clave para su función como es el hígado (Kawase et al., 2009). Algo similar ocurre con los niveles de expresión del transportador en hígado y riñón del cerdo que son mucho más bajos que los del ratón (Schrickx and Fink-Gremmels, 2006). Además, ciertas especies presentan órganos que no existen en otras especies y que pueden jugar un papel importante en la absorción/eliminación de sustratos, como sucede con el rumen bovino, en el que recientemente se ha determinado la expresión de ABCG2 (Haslam and Simmons, 2014). Todo ello sugiere que es interesante estudiar el nivel de expresión en diferentes especies para poder predecir de manera más precisa el comportamiento de un determinado sustrato en cada caso.

El segundo factor a tener en cuenta es la especificidad de sustratos que puede presentar características diferenciales entre las especies, ya que aunque generalmente se puede considerar similar, existen variaciones notables para algunos sustratos como se ha demostrado para el ABCG2 ovino y ABCG2 bovino (Gonzalez-Lobato et al., 2014) para sustratos como el danofloxacino o el orbifloxacino. Asimismo, el perfil inhibitorio de la proteína puede ser distinto entre diferentes especies (Gonzalez-Lobato et al., 2010).

El tercer factor de incertidumbre es la presencia de polimorfismos genéticos específicos en especies veterinarias que pueden alterar de manera drástica la respuesta a fármacos. Este es un novedoso campo que aún no se ha explorado a fondo. Existen multitud de trabajos que revelan y estudian la presencia de polimorfismos en transportadores ABC en especies relevantes para el ámbito veterinario (Roulet et al., 2003; Cohen-Zinder et al., 2005; Duncan et al., 2007; Real et al., 2011b; Ramirez et al., 2011; Cecchinato et al., 2015) aunque son pocos los estudios que se han realizado con profundidad para indagar en el efecto de los mismos. Los primeros descubrimientos a este respecto se hicieron como consecuencia de la detección

de una hipersensibilidad a la ivermectina por parte de ciertos perros de la raza Border Collie. El estudio con ratones *Abcb1* a/b ^(-/-) demostró que en ausencia de este transportador la ivermectina puede tener efectos neurotóxicos que pueden resultar deletéreos. Posteriores estudios genéticos en los perros que sufrían este efecto demostraron que eran portadores de una delección de 4 pares de bases que impedía la adecuada expresión del transportador ABCB1 (Roulet et al., 2003).

En el caso de ABCG2, los estudios realizados con el ABCG2 felino ponen de manifiesto la relevancia que puede tener este transportador en la clínica veterinaria (Mealey, 2013). Los estudios que avalan la importancia de este transportador en los gatos, comenzaron con la descripción de una patología asociada con una rápida degeneración visual en estos animales que está relacionada con la inactividad del ABCG2 felino. Hasta el momento en gatos se ha determinado la existencia de cuatro mutaciones en áreas conservadas del gen que codifica para el transportador lo cual tiene efectos dramáticos sobre su correcto funcionamiento. Gracias a la clonación estable del ABCG2 felino en células HEK293 se pudo determinar que dichas variaciones genéticas inactivan el funcionamiento del transportador. En concordancia con el perfil protector que caracteriza a esta proteína, ésta también se expresa en la barrera hemato-retinal impidiendo la entrada de sus sustratos en la retina. Los antibióticos fluoroquinolónicos son compuestos fotorreactivos, es decir que se transforman en especies reactivas tóxicas en presencia de luz. En aquellos casos en los que el transportador ABCG2 es parcialmente inactivo, como en el caso del ABCG2 felino, sucede que la proteína no es capaz de proteger a la retina de la entrada de fluoroquinolonas con lo que se termina produciendo un proceso degenerativo de la visión en los gatos que han sido tratados con estos antimicrobianos (Ramirez et al., 2011). El funcionamiento del transportador que permite la acumulación de toxinas y causa la degeneración retinal, va unido al hecho de que algunas formas sulfatadas de determinados fármacos no son reconocidas de forma apropiada por el ABCG2 felino. Como consecuencia, la dosis tóxica de acetaminofeno para los gatos (que

principalmente se elimina en forma de acetaminofeno-sulfato) es entre 3 y 5 veces menor que en humanos o perros. Por otra parte, la acumulación de cuerpos de Heinz en los eritrocitos es más común en gatos que en otras especies lo cual también puede estar asociado a una predisposición a la acumulación del grupo hemo oxidado en los eritrocitos felinos dada la imposibilidad por parte de ABCG2 de retirar este compuesto de los eritrocitos (Mealey, 2012).

Otro de los polimorfismos del transportador ABCG2, relacionado con el campo veterinario es el polimorfismo bovino Y581S descrito por Cohen-Zinder et al., (2005), al que daremos una amplia cobertura en los siguientes apartados. Dado el desarrollo exponencial de técnicas de secuenciación masiva más asequibles económicamente y más rápidas, cada vez se conocen más detalles del genoma de especies relacionadas con el ámbito veterinario. Sin embargo, el volumen de estudios en esta área de investigación aún es bajo y en lo que respecta a los polimorfismos veterinarios de ABCG2 sólo existen algunos trabajos en félidos y bóvidos.

3.6.1. Transporte de compuestos a leche mediante ABCG2 en rumiantes

Como hemos comentado anteriormente, además de la expresión en órganos como el hígado, el riñón y el intestino delgado también se descubrió que la expresión de este transportador se inducía en la glándula mamaria de ratones, humanos, vacas, ovejas y cabras durante la lactación (Jonker et al., 2005; Lindner et al., 2013).

Como consecuencia de ello, las toxinas que potencialmente están en la circulación materna pueden ser secretadas activamente a la leche exponiendo a crías y consumidores de leche a dichas sustancias.

Más allá del componente básico que rodea a esta línea de investigación, existe otro componente aplicado de gran relevancia. Uno de los principales problemas de la industria láctea es la detección y el análisis de xenotoxinas en leche que supone la retirada de la leche de la cadena alimentaria y lo que ocasiona pérdidas millonarias para el sector dado que el

consumo de productos lácteos derivados de rumiantes es un hábito alimentario generalizado. Por ello la comprensión y el manejo de aquellos sistemas biológicos que estén directamente implicados en la concentración de estos compuestos en leche se deben considerar como un importante valor añadido a la investigación. Además, existe una elevada presión sobre la industria láctea para obtener productos cada vez más seguros y con propiedades que puedan mejorar la salud humana.

En un proceso de producción habitual, es común que los animales tengan que ser tratados con fármacos, principalmente antibióticos, para el tratamiento de enfermedades o como medida preventiva. Existe un amplio marco legislativo cuyo objetivo es impedir la presencia de contaminantes en el producto final que llega a los consumidores. Para ello, se determinan los períodos de supresión, tiempo durante el cual el ganadero debe eliminar la leche procedente de los animales tratados con fármacos, con la consiguiente pérdida económica para el productor. A pesar de estas medidas legislativas, tras los tratamientos aún es posible encontrar en la leche de consumo, compuestos como pesticidas, dioxinas o micotoxinas, lo cual supone un riesgo para los consumidores (Wassermann et al., 2013b). Concretamente, el uso de compuestos antimicrobianos en ganadería puede incrementar el riesgo de desarrollo de resistencias bacterianas en humanos. Además, la presencia de concentraciones traza de antibióticos puede inhibir el crecimiento de bacterias lácticas necesarias para una correcta producción de otros derivados lácteos (Gonzalez-Lobato et al., 2014). Es por ello, que de manera reciente se ha abierto un nuevo campo de investigación en el que trabajan varios grupos que intenta controlar y estudiar la relevancia del transportador ABCG2 en esta área.

Anteriormente nuestro grupo ha demostrado *in vivo* en rumiantes la capacidad de reducir la secreción de fármacos antimicrobianos en leche de oveja a través de la coadministración de fármacos inhibidores de ABCG2 como los antiparasitarios ivermectina, albendazol sulfóxido o triclabendazol (Pulido et al., 2006; Real et al., 2011a; Perez et al., 2013; Barrera et al., 2013). Asimismo, también se han realizado estudios que han demostrado que dietas

enriquecidas en polifenoles procedentes de plantas, inhibidores de origen natural de la proteína, además de aumentar la cantidad de estos compuestos con actividad fitoestrogénica en leche pueden reducir la secreción de antibióticos de manera más eficaz y segura que los tratamientos agudos con inhibidores de origen farmacológico (Pulido et al., 2006; Perez et al., 2009).

La amplia gama de sustratos que reconoce el transportador involucra no sólo a carcinógenos y fármacos, también a compuestos endógenos o naturales que potencian propiedades de la leche. El enriquecimiento de los productos lácteos con moléculas bioactivas es un campo con un gran potencial para la industria. van Herwaarden et al. (2007) demostraron que la proteína ABCG2 es capaz de transportar la vitamina B₂ a la leche. La presencia de vitaminas en la leche es un factor que despierta un gran interés desde un punto de vista comercial. El estudio en profundidad de los mecanismos que enriquecen la leche en compuestos endógenos/nutrientes naturales es un campo aun sin explorar, sin embargo, cada vez son más los sustratos naturales que muestran tener la capacidad de interaccionar con este transportador, como el ácido fólico o el ácido úrico. La gama de moléculas que se relacionan con el transportador y la secreción a leche aumenta si atendemos al caso de la vitamina K₃ cuyo transporte *in vitro* ha sido demostrado pero que carece de un fenotipo *in vivo*, o al estudio de la vitamina H y la vitamina B₇ con unos niveles en leche de ratones *Abcg2*^(-/-) más bajos que en ratones *wild-type* pero cuyo transporte no se ha podido demostrar aún *in vitro* (Vlaming et al., 2009).

Previamente a los estudios *in vivo* es necesario el desarrollo de herramientas que *in vitro* nos permitan predecir la eficacia y el papel de ABCG2 en el transporte de determinados compuestos. Aunque es una proteína muy conservada filogenéticamente, diferentes grupos de investigación han optado por la clonación de las distintas isoformas del transportador que se pueden encontrar en diferentes especies de rumiantes (Real et al., 2011b; Wassermann et al., 2013b; Gonzalez-Lobato et al., 2014) (Figura 6). En este sentido el modelo *in vitro* más

popular para estudiar el transporte de fármacos mediados por este transportador en rumiantes es el estudio con células polarizadas MDCK-II que expresan de manera estable el transportador. Este modelo ha sido tradicionalmente utilizado para el estudio del transporte transepitelial de sustratos de ABCG2 murino y humano (Merino et al., 2005b; Merino et al., 2006), y de manera más reciente bovino, caprino, ovino (Real et al., 2011b; Wassermann et al., 2013b; Gonzalez-Lobato et al., 2014). Recientemente, y de manera específica para la glándula mamaria se ha puesto a punto un modelo predictivo de transporte de sustancias mediado por ABCG2 en las células HC11 que derivan del epitelio mamario y presentan una elevada expresión de dicho transportador (Tallkvist et al., 2015). Hasta el momento, los modelos celulares utilizados han demostrado gran potencialidad y capacidad predictiva del comportamiento de ABCG2 en lo que respecta a la secreción de fármacos a leche, a su inhibición y modulación (Pulido et al., 2006; Real et al., 2011b; Wassermann et al., 2013a). Además dado que existen algunos compuestos para los cuales no existen técnicas de detección suficientemente sensibles y específicas, estas herramientas se presentan como una alternativa interesante que puede ayudar a identificar aquellos fármacos que pueden aumentar su concentración en leche al ser sustratos de ABCG2.

Figura 6. Predicción de la estructura peptídica del ABCG2 bovino, caprino y ovino (Wassermann et al., 2013a)

3.6.2. Polimorfismo Y581S del ABCG2 bovino (bABCG2)

En 2005, Cohen-Zinder et al. publicaron que para uno de los *quantitative trait locus* (QTL) asociado a la producción de leche, el cambio Y581S en el transportador bovino ABCG2 era el polimorfismo causal en BTA 6 (*Bos taurus autosome 6*) que producía un efecto sobre la cantidad y composición de la leche. Este cambio de nucleótido de una adenina por una citosina (A/C) en el exón 14 del gen produce un cambio de una tirosina a una serina en la proteína en la posición 581 (Y581S). Siendo más común el alelo A, los estudios apuntan a que reduce la cantidad de leche producida y aumenta la grasa y la proteína con respecto a la variante C. La variante A se ha detectado en las especies *Bos taurus* y *Bos indicus*, mientras que el alelo C sólo se encuentra en *Bos taurus* lo cual indicaría que el alelo ancestral es el A y que el polimorfismo surgiría tras la separación de ambas especies. A raíz de estos resultados Ron et al., (2006) hicieron un estudio de distribución de este polimorfismo, con lo que se determinó que para este SNP había una gran horquilla de frecuencias en las que se puede

encontrar este polimorfismo en función de la raza. Así, en la raza israelí puede llegar hasta el 20%, hasta el 7% en la frisona británica o a un 3% en la menorquina. Adicionalmente, un estudio ha descubierto que este polimorfismo de ABCG2 también produce un efecto sobre determinadas propiedades de la coagulación de la leche lo cual podría tener importancia en aquellas explotaciones dedicadas a la producción quesera (Cecchinato et al., 2015).

El efecto que produce este polimorfismo sobre la cantidad de leche y su composición planteó la cuestión de si este polimorfismo también podría afectar de alguna manera a la capacidad de transporte de sustancias, e incluso si alguna de estas sustancias que se podrían transportar con diferente eficiencia sería la responsable de la diferente producción de leche que se había descrito entre las vacas de ambas variantes. Estudios comparativos entre diferentes especies, determinaron que la zona que engloba a la posición 581 de la proteína es una región muy conservada del gen. En otras especies se ha podido encontrar bien una tirosina o bien una fenilalanina en esta posición, siendo ambos aminoácidos aromáticos de similares características. Sin embargo, el cambio por una serina supone un cambio drástico ya que éste es un aminoácido muy hidrofílico, con lo que desde un punto de vista físico-químico es un cambio que puede tener un efecto sustancial sobre el funcionamiento global de la proteína, más aun teniendo en cuenta que se trata de una región especialmente conservada (Cohen-Zinder et al., 2005). Por ello, Merino et al., (2009) decidieron clonar ambas variantes del transportador de manera transitoria y estudiar su capacidad de transporte. Estos estudios preliminares demostraron una actividad diferente en cuanto a su capacidad para transportar el agente quimioterapéutico mitoxantrona, siendo la variante Y581S más eficiente. Aunque normalmente los polimorfismos suelen mermar la capacidad de los transportadores para actuar, no es este el único polimorfismo descrito en ABCG2 que parece incrementar la eficiencia de transporte. Este efecto ha sido previamente observado en el polimorfismo humano I206L que posee una capacidad de transportar compuestos entre dos y tres veces mayor que el genotipo salvaje (Vethanayagam et al., 2005). Con estos

antecedentes y estos estudios preliminares *in vitro* se apuntaba al polimorfismo Y581S como un polimorfismo bovino que podía tener un efecto sobre los parámetros farmacocinéticos de compuestos sustratos de ABCG2. Además también se observó que ambas variantes eran inhibidas de forma diferencial por la ivermectina, lo cual es un factor a tener en cuenta en el diseño de terapias de inhibición para reducir la secreción de fármacos a leche mediada por el ABCG2 bovino (Merino et al., 2009).

Estos resultados preliminares, condujeron en 2011 a la transducción estable de la variante bovina salvaje y de la variante polimórfica Y581S para llevar a cabo estudios comparativos que permitieran un mayor grado de complejidad experimental a la hora de estudiar las diferencias entre las dos variantes en su interacción con los compuestos que actúan como sustratos y/o inhibidores (Real et al., 2011b). En dicho estudio se describieron tres antibióticos de uso veterinario como sustratos del transportador; las fluoroquinolonas danofloxacino, enrofloxacino y difloxacino. En concordancia con los resultados publicados por Merino et al. (2009), se observó que la variante polimórfica Y581S era más activa en cuanto a su capacidad de transportar sustratos.

Estos resultados *in vitro* podrían tener implicaciones *in vivo* a nivel de absorción intestinal, hepatobiliar, renal y de secreción de leche. Además cualquier modificación farmacocinética podría afectar a los periodos de supresión establecidos hasta el momento ya que se podrían dar diferencias en lo que respecta a la eliminación del fármaco y su secreción a leche, con consecuencias para la salud pública y con repercusiones económicas para los ganaderos y la industria láctea. Un incremento en la actividad puede conllevar una mayor secreción en leche tanto de fármacos como de toxinas, y pudiendo ocurrir incluso en los productos en los que se ha respetado los periodos de supresión, lo cual tendría consecuencias para la industria láctea debiéndose ser considerado por las autoridades sanitarias.

Finalmente, la diferente capacidad de ambas variantes para ser inhibidas representa una estrategia interesante que hay que estudiar cautelosamente para abrir la puerta a una modulación específica frente a la secreción de antimicrobianos a leche.

4. OBJETIVOS

Estos antecedentes han puesto de manifiesto la función del transportador ABCG2, señalando de forma específica las posibles implicaciones para la industria láctea derivadas de la interacción de fármacos y compuestos naturales en la glándula mamaria con el transportador ABCG2 de rumiantes y en concreto el efecto del polimorfismo bovino Y581S de ABCG2. Por ello, la presente memoria tiene como objetivo general la caracterización del efecto de este polimorfismo bovino *in vivo* así como la determinación de los efectos de dietas específicas sobre la secreción en leche de fármacos mediada por ABCG2 de rumiantes. Este objetivo general se desarrollará en los siguientes objetivos específicos:

- Estudiar el efecto del polimorfismo bovino Y581S de ABCG2 sobre el transporte transepitelial de antimicrobianos como el danofloxacino y el ciprofloxacino, compuestos endógenos como el ácido úrico y la riboflavina y compuestos presentes en la dieta como la enterolactona, utilizando células que sobreexpresan ambas variantes del transportador.
- Estudiar el efecto del polimorfismo Y581S de ABCG2 bovino sobre la secreción a leche de los compuestos previamente testados en estudios de transporte transepitelial, utilizando vacas portadoras y no portadoras del polimorfismo.
- Analizar la expresión a nivel de mRNA (qRT-PCR) y proteína de ambas variantes del transportador ABCG2 bovino en células epiteliales de glándula mamaria según el genotipo, para poder interpretar de manera más adecuada los resultados derivados del estudio del polimorfismo bovino Y581S de ABCG2.
- Comprobar la interacción de la enterolactona con el transportador humano y murino tanto *in vitro* como *in vivo*, utilizando células que sobreexpresan Abcg2 murino y ABCG2 humano y ratones Abcg2^(-/-).
- Estudiar el efecto de dietas enriquecidas en semilla de lino (ricas en precursores de la enterolactona) sobre la secreción a leche de danofloxacino en ovejas.

5. RESULTADOS

5.1. ARTICLE I

The bovine ATP-binding cassette transporter ABCG2 Tyr581Ser single nucleotide polymorphism increases milk secretion of the fluoroquinolone danofloxacin.

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Short Communication

The Bovine ATP-Binding Cassette Transporter ABCG2 Tyr581Ser Single-Nucleotide Polymorphism Increases Milk Secretion of the Fluoroquinolone Danofloxacin

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ABSTRACT

The bovine adenosine triphosphate-binding cassette transporter G2 (ABCG2/breast cancer resistance protein) polymorphism Tyr581Ser (Y581S) has recently been shown to increase *in vitro* transepithelial transport of antibiotics. Since this transporter has been extensively related to the active secretion of drugs into milk, the potential *in vivo* effect of this polymorphism on secretion of xenobiotics in livestock could have striking consequences for milk production, the dairy industry, and public health. Our purpose was to study the *in vivo* effect of this polymorphism on the secretion of danofloxacin, a widely used veterinary antibiotic, into milk. Danofloxacin (1.25 mg/kg) was administered to six Y/Y 581 homozygous and six Y/S 581 heterozygous lactating cows, and plasma and milk samples were collected and analyzed by high-performance liquid chromatography. No differences were found in the pharmacokinetic

parameters of danofloxacin in plasma between the two groups of animals. In contrast, Y/S heterozygous cows showed a 2-fold increase in danofloxacin levels in milk. In addition, the pharmacokinetic elimination parameters, mean residence time and elimination half-life, were significantly lower in the milk of the animals carrying the Y/S polymorphism. These *in vivo* results are in agreement with our previously published *in vitro* data, which showed a greater capacity of the S581 variant in accumulation assays, and demonstrate, for the first time, an important effect of the Y581S single-nucleotide polymorphism on antibiotic secretion into cow milk. These findings could be extended to other ABCG2 substrates, and may be relevant for the treatment of mastitis and for the design of accurate and novel strategies to handle milk residues.

Introduction

The ATP-binding cassette (ABC) transporter ABCG2 (breast cancer resistance protein) is expressed in a wide range of tissues and organs, including the intestine, liver, blood-brain barrier, and mammary gland (van Herwaarden and Schinkel, 2006). It affects the bioavailability of its substrates and mediates the active secretion of xenobiotics and several vitamins in milk (van Herwaarden et al., 2007). The occurrence of drug residues in milk could lead to the development of bacterial resistance, allergies, or hypersensitivity reactions in consumers (McManaman and Neville, 2003). On the other hand, effective treatments for mastitis may require a considerable transfer of drugs into milk (Escudero et al., 2007). All of these issues are of great concern for general public health, the dairy industry, and veterinary therapeutics. Hence, identification of relevant factors for the transfer of drugs into milk in livestock constitutes a priority.

Changes in the expression and/or the function of ABCG2 can lead to dramatic variations in the pharmacokinetics and secretion of its substrates into milk (Jonker et al., 2007; Ni et al., 2010). Several single-nucleotide polymorphisms (SNPs) have been studied in human ABCG2, and of these, Gln141Lys (Q141K) is one of the most important, causing

impaired urate transport which may contribute to gout (Woodward et al., 2009). There is also evidence that this SNP results in increased plasma levels of diflomotecan, fluvastatin, and simvastatin, among other chemotherapeutic agents (Morisaki et al., 2005; Keskitalo et al., 2009).

With regard to ABCG2 SNPs in the veterinary field, the discovery of a nucleotide missense adenosine/cytosine mutation encoding a replacement of tyrosine-581 with serine (Y581S) localized on the fifth extracellular region of the bovine ABCG2 transporter constitutes a crucial finding. Y581S SNP is widely spread in some bovine breeds, reaching a frequency of 20% in the Israeli Holstein population (Ron et al., 2006), and it has been suggested as the causative polymorphism of a quantitative trait locus affecting the production of milk and its protein and fat composition (Cohen-Zinder et al., 2005; Weikard et al., 2012). In addition, a correlation has been suggested between this SNP and fertility (Komisarek and Dorynek, 2009). With regard to the effect of this SNP on drug transport, our previous *in vitro* results described the Y581S SNP as a gain-of-function polymorphism, showing a lower mitoxantrone accumulation in ovine primary fibroblast transiently transfected with the S581 variant, when compared with the Y581 variant (Merino et al., 2009). Recently, the greater activity of the S581 allele was confirmed on transepithelial transport of antibiotics using stably transduced cell models (Real et al., 2011a). The present study compares plasma and milk pharmacokinetics in Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows that received danofloxacin, a widely used antibiotic for the treatment of infections of the respiratory tract, intestinal tract, and mammary glands (Poutrel et al.,

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ABBREVIATIONS: ABC, ATP-binding cassette; ABCG2, adenosine triphosphate-binding cassette transporter G2; AUC, area under the curve; HPLC, high-performance liquid chromatography; Q141K, Gln141Lys; SNP, single-nucleotide polymorphism; Y581S, Tyr581Ser.

2008; Schrickx and Fink-Gremmels, 2008). The aim of our study is to show, for the first time, the potential in vivo applicability of our previous in vitro results relative to the effect of Y581S SNP on drug secretion into milk in livestock.

Materials and Methods

Reagents and Chemicals. For the pharmacokinetic studies, danofloxacin (Advocin 2.5%) was obtained from Pfizer (Cedex, France). All other chemicals were analytical grade and available from commercial sources.

Animals. Animals were housed and handled according to institutional guidelines, in compliance with European legislation (European Commission, 1986). Lactating Holstein cows aged 2 to 5 years (600–800 kg) were used. Routine milking of the cows was undertaken twice daily. The animals were parasite-free. Drinking water was available ad libitum for all animals. The experiments were performed on the private Garfi SAT farm located at Santa María del Monte del Condado, in the Province of Leon (Spain).

Cow Genotyping. DNA for animal genotyping was isolated from hair follicles using Chellex 100 (Sigma-Aldrich, St. Louis, MO) (Walsh et al., 1991) or from peripheral blood using the standard phenol-chloroform procedure. Genotypes were determined with the polymerase chain reaction–restriction fragment length polymorphism method described by Komisarek and Dorynek (2009).

Pharmacokinetic Experiments with Lactating Cows. Animals were divided into two groups of six individuals each: Y/S 581 heterozygous genotype carriers and Y/Y 581 homozygous animals. Both groups received a single dose of 1.25 mg/kg i.m. of danofloxacin (Advocin 2.5%). Blood samples were collected from the tail vein at 1, 2, 5, 11, 24, and 48 hours after treatment. Milk samples were collected after complete milking of the gland before the treatment and at 2, 5, 11, 24, 35, and 48 hours after treatment. Plasma was separated by centrifugation at 3000 rpm for 15 minutes, and plasma and milk samples were stored at -20°C until high-performance liquid chromatography (HPLC) analysis.

HPLC Analysis. The conditions for HPLC analysis of danofloxacin were modified according to previously published methods (Garcia, et al., 2000). Difloxacin (2.5 $\mu\text{g/ml}$) for plasma samples and ciprofloxacin (5 $\mu\text{g/ml}$) for milk samples were used as internal standards, and 600 μl of chloroform was added to each 100- μl aliquot of sample. Samples were shaken for 10 minutes, and the organic phase was separated by centrifugation at 5000g for 6 minutes and then evaporated to dryness under a nitrogen stream. The samples were resuspended in 100 μl of methanol and injected into the HPLC system, consisting of a Waters 600 pump, a Waters 717 plus autosampler, and a Waters 486 fluorescence detector (Waters Corporation, Milford, MA). Sample separation was performed on a reversed-phase column (Synergi 4 μm Hydro RP 80A; Phenomenex, Torrance, CA). The mobile phase consisted of 25 mM orthophosphoric acid (pH 3.0) and acetonitrile (80:20), and the flow rate was set to 1.6 ml/min. Sample detection was performed by fluorescence detection at 338 nm (excitation) and 425 nm (emission). Integration was performed using Millenium32 software (Waters Corporation).

Standard samples were prepared in the appropriate drug-free matrix. Interassay precision coefficients of variation were $<15\%$, and relative error (accuracy) values were $<20\%$. Limits of detection and limits of quantification were calculated as 3.3 and 10 times, respectively, the standard deviation of the signal corresponding to 10 blank solutions divided by the slope of the calibration curve. The limits of detection obtained were 0.8 ng/ml for plasma and 0.6 ng/ml for milk. The limits of quantification obtained were 2.3 ng/ml for plasma and 1.7 ng/ml for milk.

Pharmacokinetic Calculations and Statistical Analyses. Milk and plasma concentrations versus time curves after treatment of each individual were analyzed with the PK Solution 2.0 computer program (Summit Research Services, Ashland, OH) to obtain the estimated kinetic parameters. Results are reported as the mean $\pm$ S.D. Statistical analysis for significant differences was performed using the two-tailed Student's *t* test. A probability of $P < 0.05$ was considered to be statistically significant.

Results and Discussion

ABCG2 polymorphism research is pharmacologically and financially of relevance in the case of ruminants because of the involvement of this protein in the transport of xenobiotics and vitamins into milk.

In this paper, we studied the effect of the Y581S SNP of bovine ABCG2 on plasma bioavailability and secretion into milk of the widely used fluoroquinolone danofloxacin.

Danofloxacin (1.25 mg/kg) was administered to six Y/Y 581 homozygous and six Y/S 581 heterozygous lactating cows, and plasma and milk concentrations of the antibiotic after its administration were analyzed. Plasma levels (Fig. 1) and plasma pharmacokinetic parameters (Table 1) obtained for both groups of animals were very similar to those obtained by Shem-Tov et al. (1998) and Shojae Aliabadi and Lees (2003). Our results showed no significant differences according to the genotype. Thus, Y581S SNP does not affect systemic exposure [plasma area under the curve (AUC)] of danofloxacin at the administered dose. A lack of any effect from Y581S SNP on the plasma disposition of danofloxacin is a positive outcome for the therapeutics of systemic infections, since a change in plasma levels of the antibiotic would affect treatment efficacy as a result of the concentration-dependent effect of fluoroquinolones. Although, in general, ABCG2 does affect the plasma disposition of some of its substrates (Vlaming et al., 2009), no ABCG2-mediated effect in the systemic plasma profile of some ABCG2 substrates has been reported (Jonker et al., 2005; Zhou et al., 2008). Even human Q141K SNP does not affect the plasma disposition of all ABCG2 substrates (Kim et al., 2007; Adkison et al., 2008; Keskitalo et al., 2009). Furthermore, our previous studies using *Abcg2*^{-/-} mouse models and sheep have shown that plasma concentrations of danofloxacin are not affected by this transporter in these species (Real et al., 2011b).

In the case of levels and pharmacokinetic parameters in milk (Fig. 2; Table 1), our data are in agreement with those previously published by Shem-Tov et al. (1998), but only for Y/Y homozygous cows. Levels in the milk of Y/S heterozygous animals were significantly higher when compared with Y/Y homozygous animals at 5 and 11 hours (Fig. 2), and the milk C_{max} (0.88 ± 0.32 versus 1.76 ± 0.74 $\mu\text{g/ml}$, $P < 0.05$), $\text{AUC}_{(0-\infty)}$ values (6.53 ± 1.41 versus 12.73 ± 5.16 $\mu\text{g}\cdot\text{h/ml}$, $P < 0.05$), and AUC milk/plasma ratio (4.09 ± 1.07 versus 8.81 ± 3.69 , $P < 0.05$) were 2-fold higher in the animals carrying the

Fig. 1. Plasma concentrations of danofloxacin after its intramuscular administration at a dosage of 1.25 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows. The inset shows a semilog plot of the data. Plasma samples were collected at several points throughout 48 hours. Plasma levels of danofloxacin were determined by HPLC. Concentrations at 48 hours were undetectable. The results are presented as means; error bars indicate standard deviation ($n = 6$).

TABLE 1

Mean $\pm$ S.D. pharmacokinetic parameters of danofloxacin in plasma and milk after intramuscular administration at a dosage of 1.25 mg/kg in Y/Y and Y/S cows ($n = 6$)

	Y/Y 581	Y/S 581
Plasma		
AUC _(0-∞) (μg·h/ml)	1.59 $\pm$ 0.29	1.44 $\pm$ 0.12
C _{max} (μg/ml)	0.19 $\pm$ 0.02	0.21 $\pm$ 0.03
MRT (h)	5.95 $\pm$ 1.29	5.42 $\pm$ 0.33
T _{max} (h)	1.33 $\pm$ 0.47	1.50 $\pm$ 0.50
T _{1/2 el} (h)	4.12 $\pm$ 0.89	3.75 $\pm$ 0.23
Milk		
AUC _(0-∞) (μg·h/ml)	6.53 $\pm$ 1.41	12.73 $\pm$ 5.16*
C _{max} (μg/ml)	0.88 $\pm$ 0.32	1.76 $\pm$ 0.74*
MRT (h)	6.30 $\pm$ 0.32	5.72 $\pm$ 0.52*
T _{max} (h)	5.00 $\pm$ 0.00	5.00 $\pm$ 0.00
T _{1/2 el} (h)	4.36 $\pm$ 0.22	3.96 $\pm$ 0.36*
AUC milk/plasma	4.09 $\pm$ 1.07	8.81 $\pm$ 3.69*

AUC, area under the curve; MRT, mean residence time; T_{1/2 el}, elimination half-life; T_{max}, time to peak concentration.

* $P < 0.05$, significantly different between the two groups of animals.

polymorphism. In addition, the elimination parameters mean residence time (6.30 ± 0.32 versus 5.72 ± 0.52 hours, $P < 0.05$) and elimination half-life (4.36 ± 0.22 versus 3.96 ± 0.36 hours, $P < 0.05$) were significantly lower in the Y/S heterozygous animals, as compared with the Y/Y homozygous animals. The greater capacity to transfer danofloxacin into milk in the Y/S heterozygous animals indicates that Y581S SNP significantly affects the secretion of this antibiotic into milk. Our findings are in agreement with our previously published in vitro data showing the greater in vitro capacity of the S581 variant compared with the Y581 variant in terms of higher relative V_{max} values (Merino et al., 2009). Moreover, we recently reported a higher danofloxacin transport ratio in cells transfected with the S581 variant in comparison with Y581 cells (Real et al., 2011a). Withdrawal time for danofloxacin is set at 48 hours. Experiments with other veterinary ABCG2 substrates with longer withdrawal periods and greater persistence in milk need to be performed to elucidate the

therapeutic and financial impact of the effect of the S581 variant on the persistence of antibiotics in milk as a result of its greater capacity to extrude ABCG2 substrates. Our in vivo data might be extended to other ABCG2 substrates whose secretion into milk may be affected by this SNP, since differences between both variants in the in vitro transport of veterinary fluoroquinolones, such as enrofloxacin and difloxacin, have recently been reported (Real et al., 2011a). Milk residues of fluoroquinolones are particularly relevant, since these antibiotics are drugs with considerable stability when subjected to thermal treatments and cooking procedures. Consequently, they can remain in milk after dairy processing, and can thus reach consumers (Roca et al., 2010) if the withdrawal times are not fulfilled. Likewise, intermittent exposure of consumers of dairy products to low levels of fluoroquinolones can produce hypersensitivity reactions or affect the intestinal microflora via food intake (Normanno et al., 2005).

Our findings could have considerable relevance to other major fields, such as the quality of milk with respect to its vitamin composition, since some vitamins, such as riboflavin (van Herwaarden et al., 2007) are ABCG2 substrates and are secreted into milk. In addition, ABCG2-mediated transport of vitamins into milk might potentially affect the health of suckling calves. However, this effect of Y581S SNP on the concentrations of vitamins in milk remains to be addressed.

This study shows, for the first time, the in vivo effect of a bovine SNP on the secretion of a drug into milk, and confirms the relevance of ABCG2 polymorphisms, which might be used to predict milk pollutant kinetics more accurately. Despite the expression of Y581S as a heterozygous allele, previous in vitro results have been corroborated, showing a 2-fold greater capacity of Y/S animals to transfer the antibiotic danofloxacin into milk than Y/Y homozygous animals, with noteworthy consequences for veterinary therapeutics, milk residues, and public health.

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Fig. 2. Milk concentrations of danofloxacin after its intramuscular administration at a dosage of 1.25 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows. Milk samples were collected at several points throughout 48 hours. Milk levels of danofloxacin were determined by HPLC. Concentrations at 48 hours were undetectable. The results are presented as means; error bars indicate standard deviation ($n = 6$).

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5.2. ARTICLE II

The gain-of-function Y581S polymorphism of the ABCG2 transporter increases secretion into milk of danofloxacin at the therapeutic dose for mastitis treatment.

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Short communication: The gain-of-function Y581S polymorphism of the ABCG2 transporter increases secretion into milk of danofloxacin at the therapeutic dose for mastitis treatment

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ABSTRACT

The ATP-binding cassette transporter ABCG2 restricts the exposure of certain drugs and natural compounds in different tissues and organs. Its expression in the mammary gland is induced during lactation and is responsible for the active secretion of many compounds into milk, including antimicrobial agents. This particular function of ABCG2 may affect drug efficacy against mastitis and the potential presence of drug residues in the milk. Previous *in vitro* and *in vivo* studies showed increased transport of several compounds, including fluoroquinolones, by the bovine ABCG2 Y581S polymorphism. Our main purpose was to study the potential effect of this bovine ABCG2 polymorphism on the secretion into milk of the antimicrobial danofloxacin administered at the therapeutic dose of 6 mg/kg used for mastitis treatment. In addition, the effect of this polymorphism on the relative mRNA and protein levels of ABCG2 by quantitative real-time PCR and Western blot were studied. Danofloxacin 18% (6 mg/kg) was administered to 6 Y/Y homozygous and 5 Y/S heterozygous cows. Danofloxacin levels in milk and milk-to-plasma concentration ratios were almost 1.5- and 2-fold higher, respectively, in Y/S cows compared with the Y/Y cows, showing a higher capacity of this variant to transport danofloxacin into milk. Furthermore, the higher activity of this polymorphism is not linked to higher ABCG2 mRNA or protein levels. These results demonstrate the relevant effect of the Y581S polymorphism of the bovine ABCG2 transporter in the secretion into milk of danofloxacin after administration of 6 mg/kg, with potentially important consequences for mastitis treatment and for milk residue handling.

Key words: ABCG2, polymorphism, danofloxacin, milk secretion

Short Communication

Fluoroquinolones are a group of broad-spectrum antimicrobials that act against several microorganisms, including gram-positive and gram-negative bacteria. Under systemic infectious processes, treatment of dairy livestock with antibiotics may result in direct economic losses for industry, farmers, and consumers, due either to the drug withdrawal periods in milk or to the potential presence of drug residues. Conversely, effective mastitis systemic treatment requires drugs to extensively penetrate into milk (Ziv, 1980), and therefore, in this case, concentration of antimicrobials into milk is desirable.

The ABCG2 transporter is one of the main factors involved in drug transfer into milk (Jonker et al., 2005), and, in its characterization, authors have obviously sought to avoid, control, and predict drug secretion into milk mediated by this protein (González-Lobato et al., 2014). Although the induced expression in the lactating mammary gland (Jonker et al., 2005) is not exclusive for this ABC transporter (Farke et al., 2008; Mani et al., 2009; Ito et al., 2014), a clear effect of ABCG2 in the veterinary medicine field related with drug secretion into milk has been reported by several groups (Real et al., 2011a; Mealey, 2013).

Cohen-Zinder et al. (2005) described a nonsynonymous SNP (A/C, rs43702337) encoding a substitution of a Tyr by a Ser at position 581 (Y581S) of bovine ABCG2. This genetic change was reported to alter milk yield and composition (Cohen-Zinder et al., 2005; Olsen et al., 2007). Later on, we presented this SNP as an *in vitro* gain-of-function polymorphism, revealing a higher capacity of the Y581S variant to transport antimicrobials (Merino et al., 2009; Real et al., 2011b). These data were recently corroborated *in vivo*, showing that cows carrying the Y581S polymorphism resulted in a 2-fold increase in milk levels of danofloxacin administered at 1.25 mg/kg (2.5% formulation; Otero et al., 2013). However, a formulation of danofloxacin 18% was recently developed to allow the delivery of a higher dose

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Figure 1. Genotyping of the Y581S polymorphism of the bovine *ABCG2* gene by PCR-RFLP. Allele-specific genotyping of 6 homozygous Y/Y and 5 heterozygous Y/S animals.

(6 mg/kg). As fluoroquinolone antibacterial efficacy is dependent on the peak concentration obtained rather than the duration of plasma concentrations above the minimum inhibitory concentration (Rowan et al., 2004), the efficacy of the 18% formulation has advantages compared with the 2.5% formulation, as the former allows the administration of higher concentrations in a lower volume (Mestorino et al., 2009). Furthermore, this formulation has been reported as safe and effective in the treatment of pneumonia, enteritis, and mastitis with a single dose or at most 2 doses (Poutrel et al., 2008; Mestorino et al., 2009), with the drug reaching high concentrations in different ruminant species (Escudero et al., 2007), thus potentially reducing selection pressure for bacterial resistance (Martinez et al., 2006).

The effect of ABC transporters and their polymorphisms on drug pharmacokinetics and disposition can vary depending on the dose of drug administered; an effect of a specific SNP can be reported at only one dose, but not at others (Fukui et al., 2007), or even saturation of the ABC transporters can lead to non-linear pharmacokinetics (Maeda et al., 2011; Adane et al. 2012). Because a reported effect at one dose (in our case 1.25 mg/kg of danofloxacin) does not guarantee an extension of the effect to other doses (in our case 6 mg/kg), studying the effect of each transporter and SNP at each therapeutic dose is necessary. Therefore, the main aim of this work was to test the effect of the bovine Y581S SNP on secretion into milk of danofloxacin administered at 6 mg/kg (18% formulation). In addition, the effect of this SNP on the *ABCG2* mRNA and protein levels was also studied to test whether the *in vivo* effect of the polymorphic variant was associated to higher transcriptional rate or protein expression.

Animals were housed and handled according to institutional guidelines complying with European legislation (2010/63/EU). Lactating Holstein-breed cows aged 2 to 5 yr and weighing 600 to 800 kg were used. The daily milk yield averaged 41 ± 7 kg. The cows were milked twice daily. The animals were free of parasites. Drinking water was available *ad libitum* for all cows. The experiments were performed on the private farm Garfi SAT located at Santa María del Monte del Condado, Leon (Spain).

The DNA for animal genotyping was extracted from hair follicles using Chelex 100 (Walsh et al., 1991) or from peripheral blood by a standard phenol-chloroform procedure. Genotypes were determined by the PCR-RFLP method described by Komisarek and Dorynek (2009). After amplification of a 292-bp region of the *ABCG2* gene potentially containing the SNP and digestion with *Pst*I (Thermo Fisher Scientific, Madrid, Spain), only the amplified products containing the SNP were targets of the restriction enzyme, with restriction fragments obtained of 268 and 24 bp and only the 268 bp band detected. We selected 5 Y/S and 6 Y/Y lactating cows for further experiments (Figure 1); S/S animals could not be found. Because Y581S SNP frequency can reach 20% in some Holstein populations (Ron et al., 2006), the heterozygous pattern is expected to be more common than homozygosity. Thus, the use of heterozygous animals for our study would be the most realistic scenario.

Animals were divided into 2 groups of 5 Y/S and 6 Y/Y cows for experimental design. Both groups received a single dose of 6 mg/kg of danofloxacin (Advocin 18%, Pfizer, Cedex, France) subcutaneously in the neck. Blood samples were collected from the tail vein before the treatment and at 1, 5, 11, 24, 48, 72, and 96 h after danofloxacin administration. Milk samples were manually collected only from the front quarters for technical convenience at the same time points. No more sampling points were possible because of the private nature of the farm. Complete evacuation of the udder of each gland was performed to avoid a dilution effect during the normal milking routine (twice a day). Blood samples were centrifuged at $1,000 \times g$ for 15 min at room temperature; plasma was collected and plasma and milk samples were stored at -20°C until analysis.

The conditions for HPLC analysis of danofloxacin concentrations in plasma and milk were modified according to previously published methods (Cho et al., 2010). Ciprofloxacin at 1.25 $\mu\text{g/mL}$ for the plasma sample extraction and ciprofloxacin at 5 $\mu\text{g/mL}$ for the milk sample extraction was used as internal standard. We added 600 μL of chloroform to each 100 μL aliquot of sample; samples were shaken and the organic and water phases were separated by centrifugation at 5,000

$\times g$ for 6 min at room temperature and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 μ L of methanol and injected into the HPLC system consisting of a Waters 600 pump, a Waters 717 plus autosampler, a Waters 486 fluorescence detector (Waters Corporation, Milford, MA), and a C_{18} reversed-phase column (Synergi 4 mm Hydro RP 80A; Phenomenex, Torrance, CA). For plasma sample analyses, the mobile phase consisted of 25 mM orthophosphoric acid (pH 3.0) in a ratio with acetonitrile (80:20 vol/vol), and the flow rate of the mobile phase was set to 1.6 mL/min. For milk analyses, 1% triethylamine was added to 25 mM orthophosphoric acid (pH 3.0) and the flow rate was set to 1.85 mL/min. Sample detection was performed by fluorescence detection at 338 (excitation) and 425 nm (emission). Integration was performed using Millennium 32 software (Waters). The limits of detection were 6 ng/mL for plasma and 4 ng/mL for milk. The limits of quantification were 17 ng/mL for plasma and 13 ng/mL for milk. Plasma and milk concentrations for each animal were analyzed using a computer program (PK solution 2.0, Summit Research Services, Ashland, OH) to obtain the estimated pharmacokinetic parameters.

For quantitative real-time (qRT)-PCR and Western blot analysis, total RNA and protein of heterozygous and homozygous animals were isolated from milk fat globules according to previously published methods (Maningat et al., 2009; Pisanu et al., 2011) before administration of the drug. Fat globules contain crescents of mammary epithelial cells, which have been reported as a high-quality RNA and protein source (Maningat et al., 2009; Pisanu et al., 2011).

For qRT-PCR analysis, cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and oligo-dT primers, and qRT-PCR was performed using a Taq Man (Applied Biosystems)-based PCR assay, in accordance with manufacturer's instructions. Step One Plus Real-Time PCR System (Applied Biosystems) was used for thermocycling and to record changes in fluorescence. The PCR reaction was initiated with preincubation at 95°C for 20 s followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. Results were analyzed with Step One software v2.0. Relative gene expression was normalized to the expression of β -actin housekeeping gene using the threshold cycle (C_T) method, $2^{-\Delta C_T}$.

For Western blot analysis, 0.2 μ g of protein samples were resolved in an 8% SDS-polyacrylamide gel and electrotransferred to nitrocellulose Hybond membranes (GE Healthcare, Chalfont St. Giles, UK). The membranes were blocked with 1% skim milk then incubated overnight at 4°C with anti-mouse BXP-53 monoclonal antibody (Monosan, Uden, the Netherlands) in a 1:250

dilution. Secondary horseradish peroxidase-conjugated antibody and Immobilon Western kit (Millipore Corporation, Billerica, MA) were used to visualize ABCG2.

Statistical analysis for significant differences was performed using the 2-tailed Student's *t*-test. A probability of $P < 0.05$ was considered statistically significant.

Regarding plasma levels and plasma pharmacokinetic parameters (Figure 2, Table 1), although a tendency to lower area under the concentration curve ($AUC_{0-\infty}$) in the Y/S animals was shown (10.83 ± 3.38 vs. 7.88 ± 2.21 μ g·h/mL for Y/Y and Y/S cows, respectively), no significant differences ($P = 0.16$) in the different parameters between both groups were noted in our experimental conditions ($n = 5-6$). Plasma levels for both groups of animals were quite similar to those obtained in previous pharmacokinetic studies of danofloxacin in cows at 6 mg/kg (Mestorino et al., 2009).

In contrast to plasma pharmacokinetic parameters, milk pharmacokinetic parameters (Table 1) such as $AUC_{0-\infty}$ and C_{max} (maximum concentration) were more than 2-fold higher for Y/Y animals and 3-fold higher for Y/S cows than those previously published by Mestorino et al. (2009). These discrepancies could be attributed to differences in the experimental conditions between both reports, such as the different daily milk production (10–25 vs. 38–47 kg in our animals), different animal weight (500–650 vs. 600–800 kg in our study), different milk sampling procedure (from all quarters vs. from only front quarters in our case), or a likely different diet. It has been reported that differences in diet

Figure 2. Plasma concentrations of danofloxacin after its subcutaneous administration at a dosage of 6 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows genotyped for the Y581S polymorphism of the bovine *ABCG2* gene. Plasma samples were collected at several points over 96 h. Concentrations at 72 and 96 h were undetectable. The results are presented as means; error bars indicate SD. Y/Y cows $n = 6$; Y/S cows $n = 5$.

Table 1. Pharmacokinetic parameters (means $\pm$ SD) of danofloxacin in plasma and milk after subcutaneous administration at a dosage of 6 mg/kg in Y/Y ($n = 6$) and Y/S cows ($n = 5$) genotyped for the Y581S polymorphism of the bovine *ABCG2* gene

Parameter ¹	Y/Y 581	Y/S 581
Plasma		
AUC _{0-∞} ($\mu\text{g}\cdot\text{h}/\text{mL}$)	10.83 $\pm$ 3.38	7.88 $\pm$ 2.21
C _{max} ($\mu\text{g}/\text{mL}$)	0.84 $\pm$ 0.40	0.55 $\pm$ 0.22
MRT (h)	13.50 $\pm$ 1.63	14.83 $\pm$ 2.06
T _{max} (h)	3.00 $\pm$ 2.00	3.40 $\pm$ 1.95
T _{1/2 el} (h)	9.36 $\pm$ 1.13	10.28 $\pm$ 1.43
Milk		
AUC _{0-∞} ($\mu\text{g}\cdot\text{h}/\text{mL}$)	33.80 $\pm$ 7.15 ^a	49.94 $\pm$ 15.14 ^b
C _{max} ($\mu\text{g}/\text{mL}$)	2.88 $\pm$ 1.14	4.32 $\pm$ 2.36
MRT (h)	14.07 $\pm$ 0.75	13.51 $\pm$ 0.82
T _{max} (h)	7.00 $\pm$ 3.09	7.10 $\pm$ 3.28
T _{1/2 el} (h)	9.75 $\pm$ 0.52	9.36 $\pm$ 0.56
AUC Milk/plasma	3.42 $\pm$ 1.32 ^a	7.02 $\pm$ 3.12 ^b

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹AUC = area under the concentration curve; C_{max} = maximum concentration; MRT = mean residence time; T_{1/2 el} = elimination half-life; T_{max} = time to peak concentration.

composition, such as the presence of natural flavonoids, can change the amount of drug transported into milk (Pérez et al., 2009).

Regarding the genotype-driven differences in secretion of danofloxacin into milk (Figure 3, Table 1), our analyses showed that milk AUC_{0- ∞} values were 1.5-fold higher for cows carrying the polymorphism (33.80 $\pm$ 7.15 vs. 49.94 $\pm$ 15.14 $\mu\text{g}\cdot\text{h}/\text{mL}$ for Y/Y and Y/S cows, respectively; $P < 0.05$). This increase in danofloxacin levels in milk (1.5-fold) is lower than that previously observed for administration of 1.25 mg/kg (2-fold), thus illustrating some differences in the effect of the Y581S SNP depending on the administered dose.

To evaluate the effect of the transporter in the transfer of danofloxacin from plasma into milk, we calculated AUC milk-to-plasma ratios (3.42 $\pm$ 1.32 vs. 7.02 $\pm$ 3.12 for Y/Y and Y/S cows, respectively; $P < 0.05$), showing 2-fold higher values for the Y/S animals. This effect is similar to the one reported by Otero et al. (2013) after the administration of danofloxacin at 1.25 mg/kg with a 2-fold difference observed in the AUC milk-to-plasma ratios between both genotypes. Our results show that the Y581S SNP in *ABCG2* also increases the secretion into milk of danofloxacin administered at 6 mg/kg (18% solution).

One important issue in these studies regarding Y581S SNP of bovine *ABCG2* is to determine whether the higher activity of the SNP is due to a higher capacity of the variant to transfer danofloxacin into milk or to an increased expression of the transporter. Several reports suggest that certain SNP can increase the expression of their mRNA transcripts or proteins (Wojnowski and Brockmoller, 2004; Visser et al., 2014). Even for heterozygous individuals it has been reported that SNP

could alter the expression of *ABCG2* (Kobayashi et al., 2005). To check *ABCG2* mRNA and protein expression in the mammary gland of the 2 sets of cows (Y/S heterozygous and Y/Y homozygous), we performed reverse transcription-PCR and Western blot analysis from RNA and protein isolated from the milk fat globules. Variability in the relative *ABCG2* mRNA levels was very high between individuals in both groups (Figure 4A). Comparison of protein levels revealed that Y581S did not cause an increased expression on the transporter (Figure 4B). These data suggest that the Y581S SNP of the bovine *ABCG2* transporter probably acts in vivo as a gain-of-function polymorphism with higher capacity, as shown previously in MDCK-II in vitro models (Real et al., 2011b).

Antimicrobial therapy against mastitis includes intramammary application, systemic application, or a combination of both. However, a systemic application should also result in effective concentrations of antimicrobials in the mammary gland (Ziv, 1980; Schrickx and Fink-Gremmels, 2008). Therefore, knowledge of the main factors that could alter concentrations of relevant drugs in milk is essential in veterinary therapy for reasons of potential treatment failure or unexpected contamination of milk. Our findings are a new step toward the identification of factors which alter drug exposure in livestock and which are involved in interindividual variability. This will allow better adjustment of doses in such a way that they are high enough to guarantee efficacy and avoid the development of resistance in the

Figure 3. Milk concentrations of danofloxacin after its subcutaneous administration at a dosage of 6 mg/kg to Y/S 581 heterozygous and Y/Y 581 homozygous lactating cows genotyped for the Y581S polymorphism of the bovine *ABCG2* gene. Milk samples were collected at several points over 96 h. Concentrations at 72 and 96 h were undetectable. The results are presented as means; error bars indicate SD. Letter a represents significant differences ($P < 0.05$) between both genotypes. Y/Y cows $n = 6$; Y/S cows $n = 5$.

Figure 4. Relative ABCG2 mRNA (a) and protein (b) expression in cow mammary epithelial cells from Y/Y and Y/S individuals genotyped for the Y581S polymorphism of the bovine *ABCG2* gene. The results are presented as means; error bars indicate SD. In (b), optimal protein isolation from milk fat globules was only possible for 6 animals.

animal. However, in the case of systemic infections, drug transfer into milk is undesirable. Primary control of residues is by adherence to label withdrawal period statements with the subsequent economical loss for the farmer (Seegers et al., 2003; Imperiale et al., 2009).

In conclusion, increased secretion of danofloxacin administered at the therapeutic dose of 6 mg/kg in animals carrying the Y581S ABCG2 SNP could have relevant consequences in mastitis treatment. This newly discovered factor involved in interindividual disposition variability of antimicrobials could potentially affect treatment efficacy and contribute to milk contamination.

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5.3. ARTICLE III

Effect of the bovine ABCG2 Y581S polymorphism on plasma pharmacokinetics and secretion into milk of enrofloxacin and its active metabolite ciprofloxacin

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Manuscript in preparation

Original Article**Effect of the bovine ABCG2 Y581S polymorphism on plasma pharmacokinetics and secretion into milk of enrofloxacin and its active metabolite ciprofloxacin**

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Abstract

The veterinary relevance of ATP-binding cassette transporter G2 (ABCG2) and its genetic variants is based on their role in the secretion of drugs into milk as was previously shown with the bovine Y581S ABCG2 polymorphism which increased secretion into milk of the fluoroquinolone danofloxacin. Up to now, danofloxacin is the only drug tested for the in vivo effect of this polymorphism. Enrofloxacin is an antimicrobial exclusively used in veterinary medicine that is mainly biotransformed to its active metabolite ciprofloxacin. The aim of this work was to study the effect of the bovine Y581S ABCG2 polymorphism on in vitro transport, plasma pharmacokinetics and secretion into milk of enrofloxacin and ciprofloxacin.

Pharmacokinetic studies administering enrofloxacin 2.5 mg/kg subcutaneously to Y/Y 581 and Y/S 581 cows have reported that plasma $AUC_{0-\infty}$ values for both enrofloxacin and ciprofloxacin were significantly lower in Y/S animals. Regarding milk parameters, $AUC_{0-\infty}$ milk-to-plasma ratios were more than 1.5-fold and 2.5-fold higher in Y/S cows for enrofloxacin and ciprofloxacin respectively, revealing a higher capacity of Y581S to transfer these drugs into milk. Additional experiments using Abcg2^(-/-) mice and cells overexpressing bovine ABCG2 support the effect of ABCG2 and its Y581S polymorphism on the transport of these fluoroquinolones, thus demonstrating the role of ABCG2 in the secretion of enrofloxacin into milk and the more efficient in vitro transport of ciprofloxacin by the S581 compared to the Y581 variant. These results reinforce the clinical relevance of this polymorphism as a factor affecting plasma pharmacokinetics and milk secretion of relevant veterinary drugs.

Keywords: Bovine ABCG2; polymorphism; enrofloxacin; ciprofloxacin; transport

Introduction

Enrofloxacin and its active metabolite ciprofloxacin are broad spectrum antimicrobials belonging to the group of fluoroquinolones. Enrofloxacin is indicated in cattle for the treatment of respiratory and alimentary tract diseases of bacterial or mycoplasmal origin at a daily dose rate of 2.5 mg/kg. Recommendations have been made to increase the dose to 5 mg/kg for some bacterial infections (McKellar et al., 1999). Enrofloxacin is rapidly biotransformed to different metabolites after administration (Lopez-Cadenas et al., 2013). Ciprofloxacin is its main active metabolite, widely used as an antimicrobial in human medicine (Alshammari et al., 2014). Previous studies have shown high penetration into cow milk of ciprofloxacin after enrofloxacin administration (Idowu et al., 2010; Marin et al., 2010).

Currently milk contamination by drugs administered to livestock is an increasing concern due to economic losses and the development of antibiotic resistance from potential drug residues. The maximum residue limit for enrofloxacin (as the sum of enrofloxacin and ciprofloxacin) in milk is 100 µg/kg (Commission Regulation EC No. 1181/2002). Adherence to label withdrawal period statements together with a deep knowledge of the factors involved in drug milk secretion may provide solutions to this problem. Several fluoroquinolones such as ciprofloxacin or danofloxacin have been reported to be secreted into milk by the ABCG2 transporter using *Abcg2*^(-/-) mice (Merino et al., 2006; Real et al., 2011a). This transporter belongs to the ABC transporter family, a group of proteins that usually play a protective role in cells and tissues against toxic compounds and limit the organism exposure to potentially noxious molecules (Petzinger and Geyer, 2006). Several in vivo studies have indicated that ABCG2 mediates intestinal, hepatobiliary and renal excretion of its substrates (reviewed by Jani

et al., (2014)) affecting drug efficacy, drug–drug interactions and drug adverse effects (Ballent et al., 2012; Mealey, 2013). In 2005 Jonker et al., (2005) showed that expression of ABCG2 is induced during lactation and plays an important role in secreting drugs into mouse milk.

In the veterinary field, enrofloxacin was the first veterinary fluoroquinolone to be identified as an in vitro substrate of ABCG2 (Pulido et al., 2006). Its concomitant administration in vivo with ABCG2 inhibitors decreased enrofloxacin concentrations in sheep milk (Pulido et al., 2006). In cattle, the genetic variant Y581S ABCG2 has been reported as an in vitro and in vivo gain-of-function polymorphism with a higher capacity to transport compounds in vitro, including enrofloxacin (Real et al., 2011b). Administration of danofloxacin to cows carrying the Y581S polymorphism resulted in higher milk levels of this fluoroquinolone compared to non-carrier animals (Otero et al., 2013, 2014).

Due to its considerable relevance in veterinary medicine we considered it of interest to study the effect of the Y581S polymorphism of bovine ABCG2 on the plasma pharmacokinetics and secretion into milk of enrofloxacin and ciprofloxacin by administering enrofloxacin to Y/Y 581 and Y/S 581 cows. Additional experiments in support of this study were the assessment of enrofloxacin secretion into milk mediated by ABCG2 using *Abcg2*^(-/-) mice and the differential in vitro transport by both variants using cells overexpressing bovine ABCG2.

Materials and methods

Cell cultures

Madin-Darby canine kidney epithelial cell (MDCKII) parental cells were provided by Dr A.H. Schinkel (Netherlands Cancer Institute). MDCKII cells stably transduced with both bovine variants (S581 and Y581) of ABCG2 were previously generated by our group (Real et al., 2011b). Culture conditions were as previously described (González-Lobato et al., 2014).

Transport studies

Transepithelial transport assays using Transwell plates were carried out as described elsewhere (Perez et al., 2013) with minor modifications. Cells were grown for 3 days after seeding on microporous polycarbonate membrane filters at a density of 1.0×10^6 cells per well. To check the tightness of the monolayer, transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore). The appearance of ciprofloxacin (Sigma–Aldrich) in the acceptor compartment was presented as the fraction of total ciprofloxacin added to the donor compartment at the beginning of the experiment.

Animal Experiments

Animals were housed and handled according to institutional guidelines in compliance with European legislation (2010/63/EU). The experiments were approved by the Research Committee for Animal Use of the University of Leon (approval number 13-2011, date of approval 7 November 2011).

Milk Secretion Studies in Mice

Lactating female *Abcg2*^(-/-) and wild-type mice, all of 99% FVB genetic background and between 9 and 13 weeks of age were used. Animals were kindly provided by Dr. A.H. Schinkel (The Netherlands Cancer Institute) and kept in a temperature-controlled environment with a 12-h-light/12-h-dark cycle.

Pups of approximately 10 days old were separated from the mother 4 h before the experiment was started. To stimulate milk secretion, oxytocin (200 µl of 1 IU/ml solution) was administered SC to lactating dams. Enrofloxacin (5 mg/kg, Sequoia Research Products) was injected into the tail vein, and milk was collected at 20 min from the mammary glands by gentle vacuum suction. Blood samples were collected by orbital bleeding under anesthesia with isoflurane. At the end of the experiment mice were killed by cervical dislocation. Milk and plasma were stored at -20°C until the HPLC analysis.

Pharmacokinetic Studies in Cows

Lactating Holstein-breed cows aged 2-5 years and weighing 650–830 kg were used. The daily milk yield averaged 45 ± 8 kg. The experiments were performed on the private farm Garfi SAT located at Santa María del Monte del Condado, Leon (Spain). Y581S genotypes were determined according to Otero et al. (2014). Animals were divided into two groups: five Y/S 581 carriers and six Y/Y 581 non-carriers. Both groups received a single dose of 2.5 mg/kg of enrofloxacin SC (Baytril 10%, Bayer).

Blood samples were collected from the tail vein at 1, 5, 11, 24 and 48 h after treatment. Milk samples were manually collected at 1, 2, 5, 11, 24 and 48 h after treatment. Complete evacuation of the udder of each gland was performed to avoid a dilution effect during the normal milking routine (twice a day). No more sampling points were

possible because of the private nature of the farm. Plasma was separated by centrifugation at 1000 g for 15 min, and plasma and milk samples were stored at -20°C until high-performance liquid chromatography (HPLC) analysis.

High Performance Liquid Chromatography (HPLC) Analysis

Determination of enrofloxacin concentrations of samples from the mouse experiments were based on previously published methods (Pulido et al., 2006) with minor modifications. Sarafloxacin ($3\text{ }\mu\text{g/mL}$, Sigma-Aldrich) was used as an internal standard and samples were extracted with dichloromethane. The mobile phase consisted of 25 mM orthophosphoric acid supplemented with 0.1 % triethylamine (Merck) (pH 3.0) and acetonitrile (75:25) at a flow rate of 1 mL/min. Detection was performed with an absorbance detector at 278 nm. The limit of quantification (LOQ) was $0.3\text{ }\mu\text{g/mL}$ for plasma and milk.

Conditions for HPLC analysis of ciprofloxacin and enrofloxacin concentration from transport assays and pharmacokinetic studies in cows were modified according to previously published methods for other fluoroquinolones (Perez et al., 2013; Barrera et al., 2013). Danofloxacin ($0.01\text{ }\mu\text{g/mL}$, Sigma-Aldrich) for the plasma analysis and difloxacin ($0.01\text{ }\mu\text{g/mL}$, Sigma-Aldrich) for the milk analysis were used as internal standards. Samples were extracted with chloroform. Samples from the transport assays were not processed, and $10\text{ }\mu\text{L}$ of the culture media was directly injected into the HPLC system. The mobile phase consisted of 25 mM orthophosphoric acid supplemented with 0.1 % triethylamine (Merck) (pH 3.0): acetonitrile (87:13), at a flow rate of 1.25 mL/min. Fluorescence was detected at 280 nm (excitation) and 460 nm (emission). LOQ values in plasma were 3.9 ng/mL for enrofloxacin and 7.8 ng/mL for ciprofloxacin, and in milk 7.8 ng/mL for both enrofloxacin and ciprofloxacin.

Results

Secretion of enrofloxacin into milk in Abcg2^{-/-} and wild-type mice

To test whether enrofloxacin secretion into milk is mediated by ABCG2, enrofloxacin 5 mg/kg was administered IV to lactating wild-type and Abcg2^(-/-) and plasma and milk were collected after 20 min (Fig. 1). An increase in enrofloxacin secretion into milk was observed in wild-type compared to Abcg2^(-/-) mice (2.23 ± 0.20 vs. 1.74 ± 0.26 $\mu\text{g/mL}$, $P < 0.05$). Higher milk-to-plasma ratios were obtained in wildtype compared to Abcg2^(-/-) mice (4.48 ± 0.68 vs. 3.66 ± 0.54 $\mu\text{g/mL}$, $P < 0.05$), revealing the important role of Abcg2 in the active transport of enrofloxacin into milk. These experiments together with similar studies with ciprofloxacin (Merino et al., 2006) demonstrate the involvement of Abcg2 on milk secretion of enrofloxacin and ciprofloxacin into milk.

In vitro transport of ciprofloxacin by both bovine ABCG2 variants (S581, Y581)

To determine whether the bovine ABCG2 variants can differentially transport ciprofloxacin, transport assays were performed using polarized MDCKII parental cells and their transduced subclones with both bovine ABCG2 variants (S581 and Y581). Previous in vitro studies have already demonstrated that the parental drug enrofloxacin is more efficiently transported by the S581 variant (Real et al., 2011b).

Basal-to-apical transport of ciprofloxacin was highly increased in the bABCG2transduced subclones compared to parental MDCKII cells (Fig. 2), indicating a specific transport of ciprofloxacin by bovine ABCG2 due to its apical localization. Moreover, the relative transport ratio (i.e., the apical directed translocation divided by the basolateral directed translocation at $t = 4$ h) was higher for the S581 variant

compared to the Y581 variant (1.70 ± 0.22 versus 1.41 ± 0.11 , $P < 0.05$), thus showing a higher capacity of the first variant to transport ciprofloxacin. In all cases, the bovine ABCG2-mediated transport was reverted with the specific ABCG2 inhibitor Ko143. With these data we have demonstrated that the bovine ABCG2 S581 variant is able to transport ciprofloxacin more efficiently than the Y581 variant.

Effect of the Y581S bovine polymorphism in plasma and milk disposition of enrofloxacin and ciprofloxacin in cattle

To study further the in vivo effect of the Y581S polymorphism on the plasma pharmacokinetics and secretion into milk of enrofloxacin and its main active metabolite ciprofloxacin, plasma and milk concentrations were analysed after the administration of enrofloxacin at 2.5 mg/kg to Y/Y 581 and Y/S 581 cows (Figs. 3 and 4). During the genotyping procedure, S/S animals could not be found.

Area under plasma concentration curve ($AUC_{0-\infty}$) values for enrofloxacin and ciprofloxacin were around 20% lower for the Y/S cows compared to those observed in Y/Y cows for enrofloxacin and ciprofloxacin (Table 1), thus indicating that the Y581S polymorphism decreases plasma disposition of these antimicrobials.

Regarding secretion into milk, the area under milk concentration curve ($AUC_{0-\infty}$) values and $AUC_{0-\infty}$ milk-to-plasma ratios for enrofloxacin were around 1.5-fold higher for Y/S compared to Y/Y cows (Table 2). For ciprofloxacin, milk levels were significantly higher for Y/S compared to Y/Y cows at 5, 11, 24 and 48 h (Fig. 4). The milk maximum concentration (C_{max}) and $AUC_{0-\infty}$ for ciprofloxacin were 2-fold higher in

the cows carrying the Y581S polymorphism compared to non-carriers (Table 2). Moreover AUC_{0-∞} milk-to-plasma ratios of ciprofloxacin were more than 2.5-fold higher in Y/S animals. These results indicate that the Y581S polymorphism increases the secretion into milk of ciprofloxacin and enrofloxacin.

Discussion

It is widely known that genetic variants of human ABCG2 can lead to altered drug pharmacokinetics, affect therapy response and clinical outcome and be associated with disease states such as gout (Bruhn and Cascorbi, 2014). In the veterinary field, previous studies have reported that the defective feline ABCG2 is responsible for fluoroquinolone-induced retinal toxicity in cats (Ramirez et al., 2011) and that bovine ABCG2 Y581S polymorphism can affect the secretion into milk of danofloxacin in cows (Otero et al., 2013, 2014). The frequency of this bovine polymorphism can reach 20% in some Holstein populations (Ron et al., 2006). In the present study, we increased the range of drugs that can be affected by this polymorphism to enrofloxacin and its active metabolite ciprofloxacin.

First of all, we tested whether enrofloxacin secretion into milk is mediated by ABCG2 using Abcg2^(-/-) mice. Secretion into milk mediated by ABCG2 in the case of other fluoroquinolones, including ciprofloxacin, has been previously demonstrated with mouse models (Merino et al., 2006; Real et al., 2011a). However, the actual role of ABCG2 in milk secretion of enrofloxacin was not previously known. According to our experiments, enrofloxacin was secreted into milk more efficiently by the wild-type than

the *Abcg2*^(-/-) mice (Fig. 1). These results evidenced the role of ABCG2 in the secretion into milk of enrofloxacin.

To study the effect of bovine ABCG2 Y581S SNP on transport, plasma pharmacokinetics and secretion into milk of enrofloxacin and ciprofloxacin, we first checked the effect of this SNP on transcellular transport in cells overexpressing the bovine variants and then in vivo with cows carrying the polymorphism. In vitro results showed for the first time that bovine ABCG2 extrudes and therefore interacts with ciprofloxacin in a specific manner (Fig 2). As previously shown for enrofloxacin (Real et al., 2011b), cells overexpressing the S581 variant transport ciprofloxacin in the basolateral to apical direction more efficiently than the cells with the Y581 variant, indicating a higher transport capacity in vitro for this variant, which is in accordance with the results obtained previously for other drugs such as difloxacin, danofloxacin, marbofloxacin and nitrofurantoin (Real et al., 2011b, González-Lobato et al., 2014).

The in vivo effect of bovine ABCG2 Y581S polymorphism on enrofloxacin and ciprofloxacin disposition was confirmed in an in vivo setting when enrofloxacin (Baytril 10%) was administered to five Y/S 581 and six Y/Y 581 cows. Plasma pharmacokinetic parameters obtained for enrofloxacin and ciprofloxacin (Table 1) were in the same range as those obtained by McKellar et al. (1999). These plasma concentrations were above the in vitro minimal inhibitory concentrations for important pathogenic organisms (McKellar et al., 1999). Regarding the genotype-driven effect, plasma AUC_{0-∞} values for enrofloxacin were lower for Y/S than for Y/Y cows (Table 1). This is the first time that a decrease in plasma drug disposition has been reported for the bovine Y581S SNP. A tendency toward lower danofloxacin AUC_{0-∞} in the Y/S animals was previously reported but no significant differences in the different plasma parameters between both groups

were noted (Otero et al., 2014). ABCG2 was first described as a transporter involved in the absorption and excretion of drugs (Jonker et al., 2000) and its expression in liver, kidney and intestine of cattle (Zancanella et al., 2013) together with its induced expression during lactation in bovine mammary gland (Jonker et al., 2005) has been confirmed. Therefore, the decrease in plasma disposition of enrofloxacin in Y/S cows is probably due to the enhanced function of the Y581S polymorphism which may promote a higher clearance of the drug to urine, the primary via of excretion of enrofloxacin (Martinez et al., 2006), bile, intestinal lumen and milk.

Plasma $AUC_{0-\infty}$ values for ciprofloxacin were also lower for Y/S than for Y/Y cows (Table 1). However, in this case, the differences between both groups could not only be attributed to an effect of the Y581S SNP, but also in part to the metabolism of enrofloxacin that is converted to ciprofloxacin mainly in the liver (Lopez-Cadenas et al., 2013). Different levels of enrofloxacin were metabolized to ciprofloxacin in each set of cows and the lower plasma levels of ciprofloxacin in Y/S cows could be the consequence of the lower plasma levels of enrofloxacin in these animals. In fact, the differences in the plasma $AUC_{0-\infty}$ values between Y/Y and Y/S cows for both enrofloxacin and ciprofloxacin were very similar (around 20% in both cases).

Regarding genotype-driven differences in milk parameters, we also observed an effect due to the higher transport capacity of the Y581S polymorphism. The higher milk $AUC_{0-\infty}$ values and $AUC_{0-\infty}$ milk-to-plasma ratios in the Y/S compared to the Y/Y animals (Table 2) indicate a higher secretion into milk of enrofloxacin and ciprofloxacin by Y/S cows. The differences in the $AUC_{0-\infty}$ milk-to-plasma ratios between both sets of cows (1.5-fold for enrofloxacin and 2.5-fold for ciprofloxacin) were similar to those reported for danofloxacin by Otero et al. (2013, 2014) after its administration at two

different doses with a 2-fold difference observed in the $AUC_{0-\infty}$ milk-to-plasma ratios between Y/S and Y/Y animals.

$AUC_{0-\infty}$ milk-to-plasma ratios for ciprofloxacin were higher than ratios for enrofloxacin (6-fold higher for Y/Y animals and 9-fold for Y/S animals, Table 2), showing that ciprofloxacin is more efficiently accumulated into cow milk than enrofloxacin. Independently of the genotype, the antimicrobial profile in milk after enrofloxacin administration is mainly dominated by ciprofloxacin (Fig. 2). These higher milk levels of ciprofloxacin relative to enrofloxacin have been previously reported (Rantala et al., 2002; Kaartinen et al., 1995; Idowu et al., 2010). Thus, in the case of mastitis ciprofloxacin would be the key molecule acting against the infectious agent. It has been suggested that the high milk accumulation of ciprofloxacin could be due in part to a slow metabolism of enrofloxacin to ciprofloxacin in the udder and the trapping of ciprofloxacin in the milk due to its ionization in the low pH of the milk (Malbe et al., 1996). Although enrofloxacin is mainly metabolized in the liver (Lopez-Cadenas et al., 2013), it cannot be ruled out that part of the difference observed in secretion into milk of ciprofloxacin between both genotypes could be due to different enrofloxacin levels in the milk (susceptible to be metabolized to ciprofloxacin in the udder). However, the much greater differences between Y/S and Y/Y cows in $AUC_{0-\infty}$ milk-to-plasma ratio for ciprofloxacin (2.5-fold) as compared to enrofloxacin (1.5-fold) (Table 2) indicate an additional effect of the Y581S polymorphism on ciprofloxacin secretion into the milk regardless of the enrofloxacin metabolism in the udder.

Conclusions

This work highlights the relevance of the Y581S polymorphism on the pharmacokinetics in plasma and milk of enrofloxacin and its active metabolite ciprofloxacin, both of which drugs being widely present in the veterinary field. However, the effect of this polymorphism may not be restricted only to fluoroquinolones, since other ABCG2 substrate drugs could also be affected. Our findings provide evidence that genetic factors such as this polymorphism must be taken into account when designing accurate veterinary therapies since they can alter the expected drug profile in plasma and milk and may lead to treatment failure or resistance development due to potential drug residues in milk.

Conflict of interest statement

The authors declared no conflict of interest

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Table 1

Plasma pharmacokinetic parameters (means $\pm$ SD) of enrofloxacin and ciprofloxacin after SC administration of enrofloxacin at a dosage of 2.5 mg/kg in Y/Y (n = 6) and Y/S

Parameter	Enrofloxacin		Ciprofloxacin	
	Y/Y	Y/S	Y/Y	Y/S
AUC_{0-∞} (μg·h/mL)	2.33 $\pm$ 0.23	1.86 $\pm$ 0.32*	2.01 $\pm$ 0.08	1.71 $\pm$ 0.20*
C_{max} (μg/mL)	0.23 $\pm$ 0.04	0.20 $\pm$ 0.03	0.10 $\pm$ 0.02	0.09 $\pm$ 0.02
MRT (h)	6.92 $\pm$ 0.85	7.07 $\pm$ 0.55	16.57 $\pm$ 3.76	18.61 $\pm$ 3.58
T_{max} (h)	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	2.30 $\pm$ 1.88	2.60 $\pm$ 1.90
T_{1/2 el} (h)	5.15 $\pm$ 0.51	5.73 $\pm$ 0.49	11.75 $\pm$ 2.88	12.95 $\pm$ 2.12

cows (n = 5).

* Significantly different from the Y/Y group ($P < 0.05$).

AUC, area under the concentration curve; C_{max}, maximum concentration; MRT, mean residence time; T_{1/2 el}, elimination half-life; T_{max}, time to peak concentration.

Table 2

Milk pharmacokinetic parameters (means $\pm$ SD) of enrofloxacin and ciprofloxacin after SC administration of enrofloxacin at a dosage of 2.5 mg/kg in Y/Y (n = 6) and Y/S

Parameter	Enrofloxacin		Ciprofloxacin	
	Y/Y	Y/S	Y/Y	Y/S
AUC_{0-∞} (μg·h/mL)	1.23 $\pm$ 0.08	1.55 $\pm$ 0.18*	6.04 $\pm$ 2.38	13.46 $\pm$ 2.28*
C_{max} (μg/mL)	0.11 $\pm$ 0.03	0.13 $\pm$ 0.01	0.51 $\pm$ 0.24	1.03 $\pm$ 0.25*
MRT (h)	10.08 $\pm$ 4.38	9.81 $\pm$ 2.21	10.68 $\pm$ 2.35	10.88 $\pm$ 0.84
T_{max} (h)	3.00 $\pm$ 1.41	3.20 $\pm$ 1.45	5.00 $\pm$ 0.00	6.20 $\pm$ 2.40
T_{1/2 el} (h)	6.96 $\pm$ 3.20	6.95 $\pm$ 1.95	7.20 $\pm$ 1.82	7.27 $\pm$ 0.55
AUC Milk/plasma	0.52 $\pm$ 0.03	0.84 $\pm$ 0.15*	3.00 $\pm$ 1.19	7.79 $\pm$ 0.66*

cows (n = 5).

* Significantly different from the Y/Y cow group ($P < 0.05$).

AUC, area under the concentration curve; C_{max}, maximum concentration; MRT, mean residence time; T_{1/2 el}, elimination half-life; T_{max}, time to peak concentration.

Fig. 1. Plasma and milk concentration (A) and milk/plasma ratio (B) of enrofloxacin in wild-type and *Abcg2*^(-/-) lactating females. Enrofloxacin (5 mg/kg) was administered IV to mice, and milk and plasma were collected after 20 min and analyzed by HPLC. Results are means; error bars indicate standard deviation (SD). (n = 5-6; * P < 0.05)

Fig. 2. Transepithelial transport of ciprofloxacin (1 μ M) in polarized MDCKII parental (non-transduced), MDCKII-S581-ABCG2 and MDCKII-Y581-ABCG2 monolayers in the absence or presence of the specific ABCG2 inhibitor Ko143 (1 μ M). The experiment was started ($t = 0$ h) by replacing the medium in either the apical or basolateral compartment with fresh Optimem medium containing 1 μ M ciprofloxacin, with or without the inhibitor 1 μ M Ko143. Aliquots of 100 μ L were taken from the opposite compartment at $t = 2$ and 4 h and measured by HPLC. The fraction of ciprofloxacin transported to the acceptor compartment was presented as a percentage of total ciprofloxacin added to the donor compartment at the beginning of the experiment.

Results are means and error bars indicate SD ($n = 3$). “Ratio” represents the relative transport ratio (i.e., the apical directed translocation divided by the basolateral directed translocation) at $t = 4$ h. $\circ$ Translocation from the basolateral to the apical compartment. $\bullet$ Translocation from the apical to the basolateral compartment.

Fig. 3. Mean plasma concentrations of enrofloxacin and ciprofloxacin after SC administration of enrofloxacin at a dosage of 2.5 mg/kg to Y/S 581 and Y/Y 581 lactating cows. Plasma samples were collected at several points over 48 h. Concentrations at 48 h were undetectable. Error bars indicate standard deviation (SD) ($n = 5-6$). * $P < 0.05$ comparing both genotypes for ciprofloxacin. † $P < 0.05$ comparing both genotypes for enrofloxacin

Fig. 4. Mean milk concentrations of enrofloxacin and ciprofloxacin after SC administration of enrofloxacin at a dosage of 2.5 mg/kg to Y/S 581 and Y/Y 581 lactating cows. The inset shows enrofloxacin data alone. Milk samples were collected at several points over 48 h. Enrofloxacin concentrations at 48 h were undetectable. Error bars indicate standard deviation (SD) (n = 5-6). *P < 0.05 comparing both genotypes for ciprofloxacin

5.4. ARTICLE IV

Effect of bovine ABCG2 polymorphism Y581S SNP on secretion into milk of enterolactone, riboflavin and uric acid

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Effect of bovine ABCG2 polymorphism Y581S SNP on secretion into milk of enterolactone, riboflavin and uric acid

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Short title: Effect of bovine ABCG2 polymorphism Y581S SNP

Abstract

The ATP-binding cassette transporter G2/breast cancer resistance protein (ABCG2/BCRP) is an efflux protein involved in the bioavailability and milk secretion of endogenous and exogenous compounds, actively affecting milk composition. A limited number of physiological substrates have been identified. However, no studies have reported the specific effect of this polymorphism on the secretion into milk of compounds implicated in milk quality such as vitamins or endogenous compounds.

The bovine ABCG2 Y581S polymorphism is described as a gain-of-function **polymorphism** that increases milk secretion and decreases plasma levels of its substrates. This work aims to study the impact of Y581S polymorphism on plasma disposition and milk secretion of compounds such as riboflavin (vitamin B2), enterolactone, a microbiota-derived metabolite from the dietary lignan secoisolariciresinol and uric acid. *In vitro* transport of these compounds was assessed in MDCK-II cells overexpressing the bovine ABCG2 (WT-bABCG2) and its Y581S variant (Y581S-bABCG2). Plasma and milk levels were obtained from Y/Y homozygous and Y/S heterozygous cows. The results show that riboflavin was more efficiently transported *in vitro* by the Y581S variant although no differences were noted *in vivo*. Both uric acid and enterolactone were substrates *in vitro* of the bovine ABCG2 variants and were actively secreted into milk with a two-fold increase in the milk/plasma ratio for Y/S with respect to Y/Y cows. The *in vitro* ABCG2-mediated transport of the drug mitoxantrone, as a model substrate, was inhibited by enterolactone in both variants, suggesting the possible *in vivo* use of this enterolignan to reduce ABCG2-mediated milk drug transfer in cows. The Y581S variant was inhibited to a lesser extent probably due to its higher transport capacity. All these findings point to a significant role of the ABCG2 Y581S polymorphism in the milk disposition of enterolactone and the endogenous molecules riboflavin and uric acid, which could affect both milk quality and functionality.

Keywords: Transporter, polymorphism, milk secretion, dairy cow, cell culture.

Implications

ABCG2 is an efflux drug transporter expressed in biological barriers that transports xenobiotics into milk. The bovine ABCG2 Y581S genetic change is clinically relevant in the excretion of fluoroquinolones into milk, with important implications for the control of drug residues. Our results showed that this polymorphism also affects to the accumulation of antioxidants and fundamental nutrients. Thus, bovine ABCG2 and its genetic variant could act as a key participant in the development of new strategies affecting nutritional quality of the milk and its antioxidant capacity by altering the content of uric acid, the enterolactone and vitamin B2.

Introduction

ABCG2, the G2 member of the ABC protein superfamily, is a transporter that is highly expressed in the lactating mammary gland and is therefore considered to be largely responsible for the active secretion of some endogenous and exogenous compounds including drugs, into milk (van Herwaarden et al., 2007). Recently, ovine and bovine variants of ABCG2 have been cloned to facilitate understanding of the interspecies variations of the transporter function (Real et al., 2011b; Gonzalez-Lobato et al., 2014; Wassermann et al., 2013b). In these terms, (Mealey, 2012) reviewed the relevance of the interaction between drugs and ABC transporters in the veterinary field with a special mention to the reported polymorphisms. (Cohen-Zinder et al., 2005) described a non-synonymous single nucleotide polymorphism (SNP) (A/C, rs43702337) encoding a substitution of a Ser by a Tyr at aminoacidic position 581 (Y581S) of bovine ABCG2. Based on the two-dimensional topology model by Wassermann *et al.*, (2013) the Y581S polymorphism of the bovine ABCG2 is located in the extracellular loop between transmembrane domains five and six of the

76 transporter. This genetic change was reported to alter milk yield and composition
77 (Cohen-Zinder et al., 2005; Olsen et al., 2007). Y581S SNP frequency can reach
78 20% in some Holstein populations (Ron et al., 2006). Moreover this SNP has been
79 described *in vitro* as a gain-of-function polymorphism revealing a higher capacity of
80 the Y581S variant (Real et al., 2011) that produces a higher *in vivo* transport of
81 antimicrobials such as danofloxacin into milk (Otero et al., 2013, 2015). The
82 involvement of ABCG2 in milk secretion and plasma levels of endogenous
83 compounds has been extensively studied in humans. Some of these studies include
84 the effect of ABCG2 and its human SNPs on the distribution of vitamins such as folic
85 acid and vitamin K3 and uric acid (Shukla et al., 2007; Assaraf, 2006; van
86 Herwaarden et al., 2007). Regarding the Dairy Industry, interest in the study of the
87 excretion of uric acid in ruminant milk is based on the fact that uric acid is a strong
88 antioxidant similar to α -tocopherol, carotenoids and ascorbic acid, which may affect
89 milk quality due to their antioxidant properties (Larsen and Moyes2010). Diet
90 composition is another factor which alters milk nutritive capacity and its properties. A
91 significant concentration of enterolactone (a microbiota-derived metabolite from the
92 dietary lignan secoisolariciresinol) has been reported in bovine milk (Antignac et al.,
93 2004). Milk from cows fed with whole flaxseed and flaxseed meal supplements or
94 white clover grass silages have been reported to have greater concentrations of
95 enterolactone (Steinshamn et al., 2008; Petit et al., 2009). Enterolactone possesses
96 antioxidant activity and dietary intake of lignans and their derivatives, as part of a
97 healthy diet, has been associated with protection against cardiovascular disease,
98 diabetes and metabolic syndrome, cancer, oxidative stress and inflammation (
99 Adolphe et al., 2010). Recently, enterolactone has been identified as a substrate of
100 the human and murine Abcg2 (Miguel et al., 2014). Riboflavin deficiency can occur in

populations fed with diets poor in dairy products. Milk and other dairy products are considered as riboflavin-rich foods and make the greatest contributions of vitamin B2 intake. Riboflavin has been previously described as murine and human ABCG2 substrate in vitro (van Herwaarden *et al.*, 2007).

The effect of ABC transporters and their polymorphisms on drug pharmacokinetics has been previously reported, but their role in the transport of endogenous substrates, which could be directly involved in milk quality, adds intriguing new features to this research. The main aim of this work therefore was to test the effect of the Y581S SNP of the bovine ABCG2 transporter on secretion into milk of enterolactone, uric acid and riboflavin. The present study compares bovine ABCG2-mediated transport of these compounds in cell cultures transduced with bovine ABCG2 and its Y581S variant, and their levels in plasma and milk of Y/Y 581 homozygous and Y/S 581 heterozygous lactating cows.

Material and methods

Chemicals

Mitoxantrone, uric acid and riboflavin were obtained from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). Enterolactone was purchased from Toronto Research Chemicals (Ontario, Canada). Ko143 was purchased from Tocris (Bristol, United Kingdom). Ultrapure Millipore water was used for all the experiments. All the other chemicals were of analytical grade and obtained from commercial sources.

Cell cultures

MDCK-II (Madin-Darby canine kidney epithelial cell) parental cells were provided by Dr A. H. Schinkel (Netherlands Cancer Institute). MDCK-II subclones stably

transduced with the bovine ABCG2 (WT-bABCG2) and its Y581S variant (Y581S-bABCG2) were generated by our group (Real *et al.*, 2011). These transduced cell lines express bABCG2 protein at similar levels. The cells were cultured in Dulbecco's modified eagle medium (DMEM) with GlutaMAX (LifeTechnologies, Inc., Carlsbad, CA, USA), supplemented with 10% (v/v) foetal calf serum (MP Biomedicals, Solon, OH, USA), penicillin (50 U/ml) and streptomycin (50 µg/ml) (Life Technologies), at 37°C and pH 7.4 in an atmosphere with 5% CO₂ (Gonzalez-Lobato *et al.*, 2014).

Transport studies

Transport assays using Transwell plates (3.0 µm pore size, 24 mm diameter; Transwell 3414; Costar, Corning Inc. USA) at a density of 1.0×10^6 cells per well were carried out as previously described (Merino *et al.*, 2005b) with parental MDCK-II cells and their transduced subclones stably expressing the bovine ABCG2 transporter (WT-bABCG2) and its Y581S variant (Y581S-bABCG2). The experiments were performed in triplicate. Two hours before the start of the experiment, medium at both the apical (AP) and basolateral (BL) sides of the monolayer was replaced with 2 ml of transport medium without serum, and either with or without the specific ABCG2 inhibitor Ko143 (1 µM). The experiment started ($t = 0$) when medium in either the apical or basolateral compartment was replaced with fresh medium containing physiological concentrations of the compounds studied (100 µM of uric acid; 10 µM of enterolactone; 0.1 µM of riboflavin), (Larson and Moyes 2010; Miguel *et al.*, 2014; Koop *et al.*, 2014). In the case of enterolactone and uric acid, transport medium used was Opti-MEM I Reduced Serum medium (Life Technologies-Gibco 31985-047). In the case of riboflavin transport assays, transport medium consisted of Hanks' Balanced Salt solution (Sigma-Aldrich) supplemented with 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid, HEPES (25 mM), sodium bicarbonate (4 mM) and glucose (19 mM). Cells were incubated at 37°C in 5% CO₂ and 100 µl aliquots of culture media were taken at t = 2 and 4 h and stored at –20°C. Concentrations of studied compounds were subsequently determined by HPLC and mass spectrometry. The ratio BL-AP/AP-BL represents the relative transport ratio (i.e., the apically directed translocation divided by the basolaterally directed translocation) at 4 h.

Accumulation assays

In vitro accumulation assays were carried out as described previously (Pavek et al., 2005). Mitoxantrone (MXR, 10 µM) was used as fluorescence substrate of ABCG2, and enterolactone (100 and 200 µM) was used to inhibit the transporter (Miguel *et al.*, 2014). Cells were cultured in 12-well plates (20 × 10³ cells/well) in complete medium for 36 hours to subconfluence. Medium was aspirated, and cells were preincubated in prewarmed Opti-MEM medium with or without enterolactone for 60 min before adding mitoxantrone. The mixture was incubated for 1 hour at 37°C. Cells were washed in ice-cold PBS and trypsinized. Collected cells were sedimented by centrifugation and resuspended in PBS with 2.5% of fetal calf serum. Relative cellular accumulation of MXR was determined by flow cytometry using a CyAn ADP cytometer (Beckman Coulter). Excitation and emission wavelengths for mitoxantrone were 635 and 650 nm. Fluorescence of accumulated substrate in populations of at least 5,000 cells was quantified from histogram plots using the median of fluorescence (MF) as direct value and indirect values relativized to inhibition of the specific ABCG2 inhibitor Ko143 (100%). ABCG2 inhibition increases accumulation of mitoxantrone in transduced cells and thus increases MF. Possible background

fluorescence from enterolactone was checked in appropriate channels, but was found to be negligible in all cases. Flow cytometry data were processed and analyzed using WinMDI software (The Scripps Institute, West Lafayette, IN).

Animals

Animals were housed and handled according to institutional guidelines complying with European legislation (2010 / 63 / EU). Lactating Holstein-breed cows aged between two and five years and weighing 600–800 kg were used. There were no differences in age, weight or milk yield between both sets of cows. The daily milk yield averaged 41 ± 7 kg. The overall lactation period was mid-late. Animals were genotyped for the Y581S polymorphism of the bovine ABCG2 transporter (Otero et al., 2014) and grouped into five Y/S heterozygous cows and six Y/Y homozygous cows. Milk and plasma sampling was performed on the private farm Garfi SAT located at Santa María del Monte del Condado (Leon, Spain). Sample collection (one per animal) was performed early in the morning. Blood samples were collected from the tail vein. Milk samples were manually collected. Blood samples were centrifuged at 1000 g for 15 min and plasma was collected. Plasma and milk samples were stored at -20°C until analysis.

Analysis by UPLC-ESI-QTOF-MS/MS of enterolactone

Plasma and milk samples (100 μl) were mixed with sodium acetate buffer and treated with hydrochloric acid in methanol, following the method of (Bolca et al., 2010), re-dissolved in methanol and filtered (0.22 μm) prior to injection into the LC-MS system. Qualitative and quantitative analyses were developed using an Agilent 1290 Infinity LC system coupled to the 6550 Accurate- Mass Quadrupole time-of-flight (QTOF)

(Agilent Technologies, Waldbronn, Germany) using an electrospray interface with jet stream technology. Chromatographic and mass spectrometry conditions were the same as those previously published for the analyses and quantification of enterolactone (Miguel et al., 2014). Enterolactone was identified in plasma and/or milk samples based on the accurate mass and isotopic pattern given in QTOF-MS acquisition mode, fragmentation information offered by targeted MS/MS experiments and direct comparison with an authentic standard. Enterolactone was quantified with the calibration curve obtained with its own standard, linear from 2.6 to 2 000 nM with $R^2 \geq 0.997$. Limit of detection and quantification was 0.8 and 2.6 nM, respectively. Recovery percentages were 60% in plasma and 70% in milk. Method repeatability was $\leq 5\%$ for intra-day repeatability and $\leq 10\%$ for inter-day repeatability.

Analysis by HPLC for uric acid and riboflavin

The HPLC system consisted of a Waters 600 pump, a Waters 717 plus autosampler and a Waters 2487 UV detector for uric acid analysis and a Waters 486 fluorescence detector for riboflavin analyses. Integration was performed using Millenium 32 Chromatography manager software (Waters).

Chromatographic conditions for uric acid analyses were based on those described by (Safranow et al., 2000) with minor modifications. Briefly, a Reverse Phase Phenomenex Synergy 4.6 μm 250 x 4.6 mm column was used and mobile phases consisted of trifluoroacetic acid, TFA 0.1% and methanol (99:1) and the flow rate was set to 1 ml/min. UV absorbance was measured at 292 nm. Experiments for uric acid transport were analyzed by directly injecting 50 μl of medium into the HPLC system. Standard samples in Opti-MEM medium ranged from 0.5 to 100 $\mu\text{g/ml}$. Uric acid from plasma and milk samples was extracted according to the method published by (Liu et

al., 2003) consisting of precipitating proteins from 100 µl of milk or plasma with 200 µl of perchloric acid (10%). After sample vortexing, they were centrifuged and supernatants were evaporated under nitrogen stream. Samples were reconstituted in 100 µl of methanol and immediately injected. Caffeine 50 µg/ml was used as internal standard for both plasma and milk. Standard samples ranged from 1.56 to 25 µg/ml in plasma and from 6.25 to 100 µg/ml in milk.

For riboflavin analysis, HPLC conditions were based on a previously published method (van Herwaarden et al., 2007). The mobile phase consisted of 50 mM ammonium acetate (pH 5)/methanol (60:40), the flow rate was set to 1 ml/min and a Reverse Phase Phenomenex Synergy 4.6 µm 250X4.6 mm column was used. Fluorescence was measured with excitation and emission wavelengths set at 372 and 520 nm, respectively. Samples were thawed and kept protected from light in brown Eppendorf tubes, and a sample of 50 µl of culture media was directly injected into the HPLC system. Standard samples in Transport Solution were prepared, yielding a concentration range from 0.0006 µg/ml to 0.3 µg/ml.

Statistical analysis

To compare levels of enterolactone or uric acid between Y/Y 581 homozygous and Y/S 581 heterozygous cows a two-sided unpaired Student's t-test was used throughout to assess the statistical significance of differences between the two sets of data as well as to compare WT-bABCG2 and Y581S-bABCG2 in accumulation assays. Differences were considered to be statistically significant when $P < 0.05$.

To compare between the six experimental conditions of transepithelial transport experiments of enterolactone, uric acid or riboflavin one-way ANOVA was used to test the differences in the mean of the ratios of transport using a post-hoc Bonferroni

test ($P < 0.05$). Statistical analyses were performed with the SPSS statistics software.

Results

Enterolactone: Transport and inhibitory activity of enterolactone by bovine ABCG2-transduced subclones cells. Relation with in vivo results (show Figure 1 and 2, show Table 1)

We used transduced WT-bABCG2 cells and their variant Y581S-bABCG2 to study enterolactone transport mediated by bovine ABCG2. Our results demonstrated that the efflux ratio (BL-AP/AP-BL at 4h) that measures preferential apical transport of enterolactone (10 μ M) (Table 1) in both transduced-subclones was significantly increased with respect to MDCK-II parental cells. When the selective ABCG2 inhibitor Ko143 was used, transport was completely inhibited resulting in an efflux ratio (BL-AP/AP-BL at 4h) equal to that of the MDCK-II parental cell line. Of the transduced-subclones, the apically directed translocation of enterolactone was higher for Y581S-bABCG2 cells (around 20%) compared with WT. These results confirm that enterolactone is transported by bovine ABCG2 with significant differences in the efflux ratio between two variants, the Y581S being more active than the WT variant.

Evaluation of the inhibitory activity of enterolactone was measured in accumulation assays with flow cytometry experiments. ABCG2 expression prevents MXR accumulation and the inhibition of ABCG2 will result in an accumulation of MXR in MDCK-II cells. The ability of enterolactone to reverse the reduced MXR accumulation in cells transduced with the transporter was tested in ABCG2 bovine subclones (Figure 1 A). The percentage of inhibition of the different concentrations of enterolactone for bovine wild type/Y581S-transduced cells was related to the effect of

the reference inhibitor Ko143 (set at 100% inhibition of ABCG2, Figure 1 B). Our results showed that enterolactone was able to inhibit the transporter increasing the accumulation of MXR, in a dose-dependent manner, showing high percentages of inhibition, especially in the bovine wild type transduced cells (60-80% inhibition at 100-200 μ M; Figure 1 B). This result supported the dual interaction of enterolactone, as substrate and inhibitor, with the bovine ABCG2 transporter.

The relation between in vitro and in vivo results was established by analyses of milk and plasma enterolactone concentration in non-carriers (Y/Y) and animals carrying the polymorphism (Y/S) were performed. We first determined the levels of enterolactone in plasma and milk (Figure 2 A), and then compared the milk/plasma ratio between both groups of cows (Figure 2 B). The results showed a significant decrease in plasma enterolactone concentration in Y/S animals (84.1 ± 17 nM vs 202 ± 51 nM in Y/S and Y/Y cows, respectively). In contrast, enterolactone levels in milk were higher than those obtained in plasma but similar in both set of cows, indicating a higher secretion capacity of enterolactone by the Y/S group as shown by the milk/plasma ratios (2.99 ± 0.46 for the Y/S cows as compared to 1.44 ± 0.50 in the Y/Y cows). These results support a stronger secretion of enterolactone into milk by the Y/S animals.

Uric acid: Transport of uric acid by bovine ABCG2-transduced subclones. *Milk/Plasma ratio in cows (show Table 2, show Figure 3)*

Parental and bovine transduced cell lines were grown to confluent polarized monolayers on porous membrane filters, and vectorial transport of the uric acid (100 μ M) across the monolayers was determined. The vectorial efflux transport, BL-AP/AP-BL at 4h, of uric acid showed a significant increase in the apically directed

translocation in bovine subclones with significant differences with respect to the MDCK-II parental cell line in the efflux ratios (Table 2), although no differences were obtained when both variants were compared. Apical transport was abolished in the presence of Ko143, thus corroborating that uric acid transport was mediated by bovine ABCG2 in a specific manner. Figure 3 shows the uric acid concentration in milk and plasma obtained in both sets of cows. No differences were observed for uric acid levels in plasma. Conversely, significant differences were obtained in uric acid milk levels ($421.1 \pm 54.2 \mu\text{M}$ for Y/S cows vs $254.4 \pm 26.3 \mu\text{M}$ for the Y/Y cows). Thus, the milk/plasma ratios observed for uric acid also indicate a higher secretory activity for this compound in the Y/S cows.

Riboflavin transport (Show Figure 4)

Vitamin B2 may represent an endogenous ligand for ABCG2 in the mammary gland. Our results on vectorial transport of vitamin B2 in bovine transduced cells show that parental MDCK-II cells displayed a high basolateral directed translocation (apical-basolateral, influx flow), whereas apical directed translocation was very low (basolateral-apical, efflux flow) (Figure 4). In addition to the presence of an active transepithelial absorptive riboflavin transport in the basolateral membrane, our results support the presence of another riboflavin transport mechanism mediated by ABCG2 possibly counteracting the endogenous absorptive process. Indeed, in MDCK-II cells overexpressing bovine ABCG2 the percentage of basolateral translocation or influx flow at 4 h was markedly decreased from 76 ± 0.34 in MDCK-II parent cells to 52.1 ± 2 in WT-bABCG2 cells and 32.2 ± 3.3 in Y581S-bABCG2 cells. This transport was completely reversed by Ko143, thus corroborating riboflavin transport mediated by both bovine ABCG2 variants. The results obtained show a significant increase in the

Ratio Basal-Apical/Apical-Basal from 0.060 ± 0.024 in MDCK-II parent cells vs 0.120 ± 0.024 in WT-bABCG2 and 0.223 ± 0.016 Y581S- bABCG2, resulting in a higher transport of riboflavin in the Y581S cells.

Despite these convincing *in vitro* results, no differences in riboflavin levels in milk were observed between both sets of cows ($3.17 \text{ nM} \pm 0.17$ for Y/Y cows vs $3.61 \text{ nM} \pm 0.16$ for Y/S cows), which suggests that the Y581S polymorphism does not affect riboflavin levels in milk.

Discussion

The study of the association between *in vitro* and *in vivo* results is difficult because of the complex interaction of genomic, proteomic and metabolomic factors and the important role of animal characteristics such as age, nutrition and stage of lactation in cows. In this report we examine the relevance of the Y581S polymorphism on the transport of endogenous and dietary compounds present in cow milk as previously reported for milk secretion of fluoroquinolone drugs such as danofloxacin (Otero *et al.*, 2013, 2015).

Our results show that the *in vitro* models can act as a valuable tool to test the potential role of the Y581S bovine polymorphism in the secretion of drugs (Gonzalez-Lobato *et al.*, 2014) and dietary-derived compounds such as enterolactone (Miguel *et al.*, 2014) into milk. The role of enterolactone as inhibitor of ABCG2 could have a significant impact on milk quality and the control of milk residues.

Uric Acid

The presence of some key compounds such as antioxidants and vitamins could affect milk properties with effects in the Dairy Industry. At the moment, several tools

have been developed to predict milk accumulation of some of these compounds, which might help to elucidate possible mechanisms behind the interindividual differences in milk quality. Nonetheless, the effect of the alteration of specific compounds on milk quality-related parameters has not yet been clearly established. This is the case of uric acid, a purine metabolite able to act as a potent free radical scavenger and thereby provide protection against oxidative damage (Larsen and Moyes, 2010). Therefore, the altered secretion of uric acid might contribute to the overall redox potential of milk together with vitamins such ascorbic acid or α -tocopherol (Aycicek et al., 2006). The milk uric acid concentration in the Y/S cows was significantly higher compared to the Y/Y cows (Figure 3). This result supports the function of mammary gland ABCG2/BCRP transporter in uric acid transport in milk as well as the differential role of the ABCG2 polymorphism. The uric acid concentration could increase the potential redox in milk as suggested by preliminary physico-chemical analyses of milk from both cow groups (milk redox value was significantly increased in Y/S polymorphic cows; Otero *et al.*, unpublished results). On the other hand, the role of ABCG2 in the extrarenal transport of uric acid is well established (Hosomi et al., 2012), as is the strong interaction between uric acid and the transporter as evidenced by the inhibition of ABCG2 by uric acid at clinically relevant concentrations in humans (IC_{50} value = 365 μ M) (Dankers et al., 2013).

Enterolactone

The enterolactone concentration in cows' milk agrees with the average content reported in previous studies (Antignac et al., 2004; Gagnon et al., 2009). Flax hulls increase concentration of enterolactone in bovine milk and the abundance of some

antioxidant genes in the mammary gland, which can promote an antioxidant environment and protect against oxidative stress damage in the mammary gland (Schogor et al., 2013; Cortes et al., 2012). Our *in vitro* results presented enterolactone not only as a specific and potent ABCG2 substrate but also as a compound more actively transported by the Y581S variant of the bovine ABCG2 transport compared to the WT variant. This is the first time that such an effect of the Y581S SNP is described for a natural component derived from silage intake. In dairy cows, lignans are metabolized in the rumen and are available for absorption in the small intestine as enterolignans (Petit et al., 2009). Some authors have pointed out the high concentration of enterolactone in milk rather than its low uptake (Steinshamn et al., 2008; Petit et al., 2009). Njastad *et al.*, (2014) have recently reported that factors such as re-conjugation and secretion into the rumen and intestinal wall as well as in cells from the udder could be limiting factors in the content of dietary isoflavones and lignans into milk. ABCG2 has been previously reported to be involved in intestinal conjugation and hepatic metabolism of isoflavones (Alvarez et al., 2010; Chen et al., 2014). Furthermore, ABCG2 expression in bovine rumen, intestine, liver and mammary gland has recently been reported (Haslam and Simmons 2014; Lindner et al., 2013). Plasma levels of enterolactone in Y/S 581S cows (84.10 ± 17.00 nM) were lower than those obtained for Y/Y 581 cows (202.00 ± 50.78 nM), which highlighted the role of this SNP in the plasma disposition of enterolactone. Moreover, milk/plasma ratio was much higher in Y/S cows (2.99 ± 0.46) than in Y/Y cows (1.44 ± 0.50), which corroborated the *in vitro* results obtained with the S581 variant. The Y581S SNP of the bovine ABCG2 can affect the plasma concentration of several substrates (Otero *et al.*, 2015). We hypothesize that the decrease in plasma disposition of specific substrates of ABCG2 in Y/S cows is

probably due to the enhanced function of the Y581S polymorphism. This effect may promote a higher clearance of some substrates in organs with elevated ABCG2 expression and therefore lead to an increase of concentration of these compounds in body fluids such as urine, bile, intestinal lumen and milk, reducing plasma concentration.

With respect to enterolactone inhibition of the bovine ABCG2, our results agree with previous data (Gonzalez-Sarrias et al., 2013), from murine *Abcg2* where significant inhibitions were achieved using 100-200 μ M enterolactone. These results indicate that mammalian lignans could be used for reducing withdrawal periods in ruminant milk and this would result in lower transfer of drugs and antibiotics into milk, as in the case of isoflavones when they were co-administered with enrofloxacin (Pulido et al., 2006). Enterolactone 100 μ M was able to efficiently inhibit the WT variant of the ABCG2 transporter, reaching almost 70% of inhibition. The Y581S variant was inhibited but to a lesser extent probably due to its higher transport capacity. This finding agrees with a previous report in which some compounds differentially inhibited both variants of bovine ABCG2 (Merino et al., 2009).

Riboflavin (vitamin B2)

Riboflavin was tested in our *in vitro* model since it is a micronutrient present in milk with an important role in a wide variety of cellular processes. Transport assays with polarized MDCK-II cells have allowed us to identify riboflavin as an ABCG2 substrate in cattle. Riboflavin has been previously described as murine and human ABCG2 substrate *in vitro* (van Herwaarden et al., 2007). Furthermore, *in vivo* assays in mice have shown that, on the one hand, *Abcg2* limits the bioavailability of riboflavin in plasma and tissues and, on the other hand, this vitamin is actively transported into

milk. However, no reports showing ABCG2-mediated riboflavin transport in ruminants are available so far. Our data reporting riboflavin transport mediated by bovine ABCG2, (Figure 4) showed a potential involvement of this transporter in the transfer of nutrients through bovine milk from the mother to the newborn. According to our results, there is a specific mechanism that translocates riboflavin to the basolateral compartment, which is in agreement with previous studies that have located specific transporters for vitamin B2 in the basolateral membrane (Yonezawa and Inui2013). Nevertheless, our results support the presence of another riboflavin transport mechanism mediated by ABCG2. van Herwaarden *et al.*, (2007), reported that in *abcg2* knockout mice milk secretion of riboflavin was reduced >60-fold compared to that in wild-type mice. Yet, under laboratory conditions, *abcg2* knockout mice pups showed no riboflavin deficiency due to concomitant milk secretion of its cofactor flavin adenine dinucleotide (FAD), which was not affected. These authors points out that a metabolic trapping of riboflavin by conversion to FAD are sufficiently effective to offset the riboflavin extrusion capacity of ABCG2 and thus avoid overall vitamin B2 deficiency. This compensatory mechanism for secretion of vitamin B2 equivalents into milk may differ between species and the effect of ABCG2 on this process might vary between ruminant and non-ruminant species.

The complexity of these mechanisms as well as the relation of *in vitro-in vivo* results was evident as no differences were obtained in cow. Despite increased *in vitro* transport of riboflavin by the Y581S variant compared to wild-type (Figure 4), we have not detected differences in the vitamin composition of milk from cows with and without the Y581S polymorphism. It is important to note that our *in vivo* studies were performed in heterozygous individuals for the Y581S polymorphism. Therefore, the effect of the *in vivo* expression of the gain-of-function SNP in a single allele might not

be as effective in terms of transport capacity as our *in vitro* model that overexpresses separately both variants of the bovine ABCG2 transporter.

In conclusion, the results obtained in this work show for the first time the significant role of the ABCG2 Y581S polymorphism in the transfer into milk of uric acid. Moreover, the role of enterolactone as inhibitor of ABCG2 could have a significant impact on milk quality and the control of milk residues. These findings describe this polymorphism as a new factor that may affect milk composition, chemical properties and quality with an important effect for the milk industry and consumers of dairy products. Our results support the *in vitro* model as a valuable tool but we cannot rule out other outcome using other cell models such as mammary cell lines. The impact of enterolactone for ruminant ABCG2-mediated drug secretion *in vivo* is an issue of interest that should be addressed in the future.

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Table 1 Concentration of enterolactone (10 μ M) permeated towards Apical (BL-AP transport) or Basal (AP-BL transport) compartments in cell cultures in the absence and in the presence of inhibitor.

		Time (h)	BL-AP (μ M)	AP-BL (μ M)	BL-AP/ AP-BL	P-value
Enterolactone	Parent	2	1.13	1.17		
		4	2.11	1.52	1.42 ^A	
	WT- bABCG2	2	3.52	1.23		
		4	3.75	1.09	3.48 ^{A,B}	0.0003
	Y581S- bABCG2	2	4.21	0.84		
		4	4.37	0.80	5.45 ^{B,C}	≤ 0.0004
	Parent	2	1.73	1.18		
		4	2.79	2.87	0.97 ^A	1.0000
Enterolactone + Ko143 (1 μ M)	WT- bABCG2	2	2.80	1.48		
		4	2.13	2.28	0.92 ^A	1.0000
	Y581S- bABCG2	2	2.17	1.60		
		4	2.43	2.55	0.99 ^A	1.0000

Results are expressed as mean. (n = 3).

Residual error term resulting from the ANOVA = 0.216.

^{A,B,C} Mean ratio values within a column not sharing the same superscripts differ significantly at P < 0.01

Table 2 Concentration of uric acid (100 μM) permeated towards Apical (BL-AP transport) or Basal (AP-BL transport) compartments in cell cultures in the absence and in the presence of inhibitor.

		Time (h)	BL-AP (μM)	AP-BL (μM)	BL-AP/AP-BL	P-value
Uric acid	Parent	2	4.14	2.88		
		4	6.31	5.98	1.14 ^a	
	WT-bABCG2	2	4.08	2.55		
		4	12.76	6.07	2.13 ^{a,b}	0.0003
	Y581S-bABCG2	2	3.43	2.64		
		4	13.15	7.72	1.72 ^{a,b}	0.0328
	Parent	2	5.39	4.03		
		4	9.89	8.28	1.20 ^a	1.0000
Uric acid + Ko143 (1 μM)	WT-bABCG2	2	7.24	7.12		
		4	13.11	14.33	0.92 ^a	1.0000
	Y581S-bABCG2	2	5.26	3.72		
		4	8.84	7.42	1.19 ^a	1.0000

Results are expressed as mean. (n = 3).

Residual error term resulting from the ANOVA = 0.326

^{a,b} Mean ratio values within a column not sharing the same superscripts differ significantly at P < 0.05

Figure 1

Figure 1 Effect of the enterolignan enterolactone on the accumulation of mitoxantrone (at 10 μ M) in parent MDCK-II cells and in their WT bABCG2 and Y581S bABCG2-transduced subclones. Cells were pre-incubated with or without the specific ABCG2 inhibitor Ko143 (1 μ M) or enterolactone at the indicated concentrations. A) Fluorescence units, (median). B) Percentage of inhibition of the different concentrations of enterolactone. Data are expressed as mean $\pm$ s.e.m. ($n = 3$, * $P < 0.05$). Percentage of inhibition was related to the effect of the reference inhibitor Ko143 (set at 100% inhibition of ABCG2).

Figure 2

Figure 2 Milk and plasma concentration (A) and milk/plasma ratio (B) of enterolactone in YY 581 and Y/S 581 lactating cows. Results are expressed as mean $\pm$ s.e.m. ($n = 5-6$; * $P < 0.05$).

Figure 3

Figure 3 Milk and plasma concentration (A) and milk/plasma ratio (B) of uric acid in Y/Y 581 and Y/S 581 lactating cows. Results are expressed as mean $\pm$ s.e.m. ($n = 5-6$; * $P < 0.05$).

Figure 4

Figure 4 Transepithelial transport of riboflavin (0.1 μ M) in polarized parent MDCK-II and in their WT bABCG2 and Y581S bABCG2-transduced subclones in the absence or in the presence of Ko143 (1 μ M). ● Translocation from the basolateral to the apical compartment. ■ Translocation from the apical to the basolateral compartment. The fraction of riboflavin transported to the acceptor compartment was presented as a percentage of the total riboflavin added to the donor compartment at the beginning of the experiment. Results are expressed as mean $\pm$ s.e.m. ($n = 3$).

5.5. ARTICLE V

Role of ABCG2 in transport of the mammalian lignan enterolactone and its secretion into milk in *Abcg2* knockout mice.

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Short Communication

Role of ABCG2 in Transport of the Mammalian Lignan Enterolactone and its Secretion into Milk in *Abcg2* Knockout Mice

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ABSTRACT

Lignans are phytoestrogens that are metabolized by the gut microbiota to enterodiol and enterolactone, the main biologically active enterolignans. Substantial interindividual variation in plasma concentration and urinary excretion of enterolignans has been reported, this being determined, at least in part, by the intake of lignan precursors, the gut microbiota, and the host's phase 2 conjugating enzyme activity. However, the role of ATP-binding cassette (ABC) transporters in the transport and disposition of enterolactone has not been reported so far. Active transport assays using parental and Madin-Darby canine kidney epithelial cells transduced with murine and human ABCG2 showed a significant increase in apically directed translocation of enterolactone in transduced cells, which was confirmed by using the selective ABCG2 inhibitor Ko143. In addition,

enterolactone also inhibited transport of the antineoplastic agent mitoxantrone as a model substrate, with inhibition percentages of almost 40% at 200 μ M for human ABCG2. Furthermore, the endogenous levels in plasma and milk of enterolactone in wild-type and *Abcg2*^{-/-} knockout female mice were analyzed. The milk/plasma ratio decreased significantly in the *Abcg2*^{-/-} phenotype, as compared with the wild-type mouse group (0.4 ± 0.1 as against 6.4 ± 2.6). This paper is the first to report that enterolactone is a transported substrate and therefore most probably a competitive inhibitor of ABCG2, which suggests it has a role in the interindividual variations in the disposition of enterolactone and its secretion into milk. The inhibitory activity identified provides a solid basis for further investigation in possible food-drug interactions.

Introduction

The ATP-binding cassette (ABC) subfamily G2 (ABCG2) protein is apically expressed ABC membrane transporter that mediates the active and outward transport of a wide range of anticancer drugs, dietary compounds, food carcinogens, and antibiotics from cells (van Herwaarden et al., 2003; Merino et al., 2005; Vlaming et al., 2009). In recent years, its role as a transporter of phytoestrogens and their conjugated metabolites has gained special relevance (Zhu et al., 2010; Alvarez et al., 2011; Tan et al., 2013).

ABCG2 is found in tumor cells, but also in the cells of a variety of normal tissues involved in the uptake and elimination of compounds, such as enterocytes, hepatocytes, and cells of the proximal tubules of the kidney. The apical localization in these cells allows ABCG2 to mediate hepatobiliary and urinary elimination and to function as a barrier to uptake from the gut lumen (van Herwaarden and Schinkel, 2006). In addition, induced expression of ABCG2 in the lactating mammary gland supports its important role in the active secretion of several xenobiotics and beneficial compounds such as vitamins into milk (Jonker et al., 2005; Merino et al., 2006; van Herwaarden et al., 2007).

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Dietary intake of lignans and their derivatives, as part of a healthy diet, has been associated with protective effects against a number of chronic diseases (Hu et al., 2007; Adolphe et al., 2010; Guglielmini et al., 2012; Högger, 2013). When consumed, plant lignans are metabolized by the gut microbiota in the upper part of the colon to enterolactone and enterodiol (Del Rio et al., 2013).

Dietary sources of plant lignans are flax grains, seeds, fruits and vegetables, olive oil, and beverages such as tea, coffee, and wine (Kuijsten et al., 2005). A significant concentration of enterolactone has been reported in bovine milk (Antignac et al., 2004). In general, under normal dietary conditions, enterolactone is the enterolignan that reaches the highest concentration in plasma (Kuijsten et al., 2006; Högger, 2013) and is the most effective metabolite in the inhibition of cell proliferation (Corsini et al., 2010; Azrad et al., 2013). Owing to the potential beneficial effects of enterolactone and the complex relationship between the colonic environment and other factors contributing to systemic exposure of such compounds to ABC transporters, a study of the interaction of ABCG2 with enterolactone could be of particular relevance. In this regard, the aim in this research was to assess the potential role of enterolactone as an ABCG2 substrate and/or inhibitor using Madin-Darby canine kidney II (MDCKII) epithelial cells transduced with the transporter. The specific *in vivo* role of the ABCG2 transporter in the disposition of this mammalian lignan was evaluated using wild-type and *Abcg2*^{-/-} knockout female mice through determination of the endogenous enterolactone milk/plasma ratio found in the two types of mice.

Materials and Methods

Chemicals. Mitoxantrone and nitrofurantoin were obtained from Sigma-Aldrich (St. Louis, MO). Enterolactone was purchased from Toronto Research

ABBREVIATIONS: ABC, ATP-binding cassette; ABCG2, ATP-binding cassette subfamily G2; MDCKII, Madin-Darby canine kidney II; MF, median of fluorescence; MXR, mitoxantrone.

Chemicals (Toronto, Ontario, Canada). All the other chemicals were of analytical grade and obtained from commercial sources.

Cell Cultures. MDCKII parent cells and their human ABCG2 and murine Abcg2-transduced subclones were kindly provided by Dr. A. H. Schinkel, Netherlands Cancer Institute (Amsterdam, The Netherlands). Culture conditions were as described in a previous publication (Merino et al., 2005). The cells were cultured in Dulbecco's modified Eagle's medium supplied with GlutaMAX (Life Technologies, Inc., Carlsbad, CA) and supplemented with penicillin (50 units/ml), streptomycin (50 μ g/ml), and 10% (v/v) fetal calf serum (MP Biomedicals, Solon, OH) at 37°C in the presence of 5% CO₂. The cells were treated with trypsin every 3 to 4 days for subculturing.

Transport Studies. Transport assays using Transwell plates were carried out as previously described (Merino et al., 2005) with minor modifications. Cells were seeded on microporous polycarbonate membrane filters (3.0- μ m pore size, 24-mm diameter; Transwell 3414; Costar, Corning, NY) at a density of 1.0×10^6 cells per well and grown for 3 days. Transepithelial resistance, measured in each well using a Millicell Electrical Resistance System Volt-Ohm Meter (Millipore, Bedford, MA), was used to check the tightness of the monolayer.

Murine Abcg2 and human ABCG2 mediated transport were determined after the addition of enterolactone to the donor compartment at the beginning of the experiment. The appearance of enterolactone in the acceptor compartment was recorded as a fraction of the total (10 μ M) added to the donor compartment at the beginning of the experiment. Aliquots of 100 μ l were taken from the opposite compartment after 2 hours and stored at -20°C until analysis.

The specific Abcg2/ABCG2 inhibitor Ko143 (Allen et al., 2002) was added concomitantly with enterolactone, to test the reliability of the efflux of enterolactone by ABCG2.

Accumulation Assays. In vitro accumulation assays were carried out as described elsewhere (Pavek et al., 2005). Mitoxantrone (MXR) (10 μ M) was used as fluorescence substrate, and enterolactone was used as an inhibitor at different concentrations. The relative cellular accumulation of MXR was determined by flow cytometry using a CyAn cytometer (Beckman Coulter, Fullerton, CA) from histogram plots using the median of fluorescence (MF). ABCG2 inhibition increases the accumulation of MXR in ABCG2-transduced cells and thus increases the MF. Inhibitory potencies were calculated as follows: Inhibitory potency = (MF with tested compound - MF without inhibitor) / (MF with Ko143 - MF without inhibitor) $\times$ 100%.

Animals. Mice (weighing 30 $\pm$ 2 g) were housed and handled in accordance with procedures approved by the Research Committee of Animal Use of the University of Leon, Spain, which were carried out under the "Principles of Laboratory Animal Care" and the European guidelines described in EC Directive 86/609. The animals used in the experiments were Abcg2^{-/-} (n = 5) and wild-type female mice (n = 5) aged between 9 and 14 weeks, all of them >99% FVB genetic background. The animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. The mice were fed normal rodent feed (Scientific Animal Food & Engineering-SAFE, A04/A04C/R04) from Panlab S.A. Barcelona, Spain, and water was available ad libitum. Pups of approximately 10 days old were separated from their mother approximately 4 hours before milk collection. Oxytocin (200 μ l of 1 IU/ml solution) was administered subcutaneously to lactating mothers to stimulate milk secretion. Blood was collected by orbital bleeding and milk was obtained from the mammary glands by gentle vacuum suction after anesthesia with isoflurane. Heparinized blood samples were centrifuged immediately at $1,000 \times g$ for 10 minutes.

Ultraperformance Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry Analysis. Plasma and milk samples (150 and 80 μ l, respectively) were mixed with sodium acetate buffer and treated with hydrochloric acid in methanol, following the method of Bolca et al., (2010), redissolved in methanol, and filtered (0.22 μ m) prior to injection into the liquid chromatography-mass spectrometry system.

Samples were analyzed using an Agilent 1290 Infinity LC system coupled to a 6550 Accurate-Mass Quadrupole Time of Flight Liquid Chromatograph/Mass Spectrometer (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology; Agilent). Data were processed using the Mass Hunter Qualitative Analysis software (B.06.00). Analytes were separated on a reverse-phase Poroshell 120 EC-C18 column (3 $\times$ 100 mm, 2.7 μ m) (Agilent Technologies) operating at 30°C. The mobile phases used were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow

rate of 0.4 ml/min and the following gradient conditions: 2% to 25% B at 0–10 minutes, 25% to 40% B at 10–16 minutes; 40% to 95% B at 16–19 minutes; and 95% to 2% at 20–21 minutes; the column was reequilibrated for 5 minutes. A volume of 2 μ l of the sample was injected. Electrospray interface parameters were as follows: gas temperature 280°C, drying gas 9 l/min, nebulizer 35 psi, sheath gas temperature 400°C, and sheath gas flow 12 l/min. Spectra were acquired in the range of 100–1,100 m/z in negative mode, and fragmentor voltage was 100 V.

Quantification of enterolactone was carried out by peak area integration of its extracted ion chromatograms at m/z 297.1132. The calibration curve was linear over the concentration range 2.6–2,000 nmol/l. The limits of detection and quantification were 0.8 nmol/l and 2.6 nmol/l, respectively. Repeatability, expressed as the relative standard deviation of peak area, was 3.3% for intraday repeatability and 6% for interday repeatability. The recovery of enterolactone from plasma and milk reached 60% and 70%, respectively.

Results and Discussion

The enterolignans enterodiol and enterolactone have been reported to exert protective effects through a variety of mechanisms, including phytoestrogenic, antioxidant, anti-inflammatory, and anticancer effects from in vitro and in vivo studies (Hu et al., 2007; Crozier et al., 2009; Högger, 2013). Recently, an inverse relationship between a decreased mortality risk and enterolactone levels >10 nmol/l have been found (Guglielmini et al., 2012).

The results obtained here give evidence for a role of ABCG2 in the transport of enterolactone. Active transport assays using parental and human ABCG2 and murine Abcg2-transduced MDCKII cells showed an increase in apically directed translocation of enterolactone in transduced cells, which was confirmed with the use of the selective ABCG2 inhibitor Ko143 (Fig. 1). These data are the first to identify the mammalian lignan enterolactone as a substrate of ABCG2. The metabolites of dietary phenolics, such as enterolignans, reach the systemic bloodstream in the range from nmol/l to low μ mol/l concentrations. These levels of bioavailable metabolites can exert modulatory effects in cells through selective actions on different components of the intracellular signaling cascades, which are vital for cellular functions such as growth, proliferation, and apoptosis (Crozier et al., 2009; Corsini et al., 2010). Therefore, the concentrations used in the present study (10 μ M/l) are physiologically relevant (Corsini et al., 2010). In addition, the metabolism and tissue distribution of enterolactone as well as its enterohepatic circulation (Högger, 2013) may be directly influenced by ABCG2 interaction. In fact, in these experiments endogenous milk/plasma concentration ratios pointed to a specific role for ABCG2 in the transport of enterolactone into milk (Table 1). The milk/plasma ratio

Fig. 1. Transepithelial transport of enterolactone (10 μ M) in MDCKII (parent), MDCKII-Abcg2, and MDCKII-ABCG2 monolayers. At 2 hours before the start of the experiment, cells were preincubated with or without the ABCG2 inhibitor Ko143 (1 μ M) (Ko). The experiment started with the addition of enterolactone to one compartment (basolateral or apical), either with or without 1 μ M Ko143. After 2 hours, the percentage of drug appearing in the opposite compartment was measured using ultraperformance liquid chromatography quadrupole time of flight mass spectrometry. The results are means; error bars indicate S.D. (n = 3 to 4).

decreased significantly in the *Abcg2*^{-/-} phenotype as compared with the wild-type mouse group (0.4 ± 0.1 as against 6.4 ± 2.6). In this regard, a noteworthy concentration of enterolactone (234 ± 90 nM) has been reported in bovine milk (Antignac et al., 2004), in agreement with the data obtained here for mouse milk (391 ± 225 nM). In addition, mean plasma concentrations of endogenous enterolactone in wild-type mice (57 ± 13 nM) (Table 1) were in agreement with the values reported in humans (Kuijsten et al., 2005; Adolphe et al., 2010). Recently, the secretion of compounds into ruminant milk has been reported to be affected by ABCG2 polymorphisms and ABCG2 inhibition by drugs and flavonoids (Real et al., 2011; Otero et al., 2013). Thus, these ABCG2-related factors presumably could affect enterolactone concentrations in milk and thus the possible health benefits of lignans from the consumption of milk. However, care should be taken since exposure to lignans during early stages of life may adversely alter normal development due to their phytoestrogen nature (Ward et al., 2001).

Enterolactone exposure is partly determined by intake of lignan precursors, intestinal bacterial activity, and phase-2 conjugating enzyme activity (Adlercreutz, 2002; Kuijsten et al., 2005; Lampe et al., 2006). Conjugation of enterolignans with sulfate and glucuronic acid occurs in the intestinal wall and liver, with the predominant conjugates being glucuronides. Both sulfate and glucuronide conjugates have been identified as ABCC2 and ABCG2 substrates (Lampe et al., 2006; Krumpochova et al., 2012; van de Wetering and Sapth, 2012). These authors found that ABCG2 was able to transport the enterolactone sulfate with low affinity, and its disposition in *Abcg2*^{-/-} mice was altered, highlighting the physiologic relevance of this result. The role of ABCG2 in the transport of phenolic compounds and their conjugates is well known (Alvarez et al., 2011). ABCG2 is not only present at a considerable level in the main organs involved in the conjugation of enterolactone, the intestinal wall and liver (Vlaming et al., 2009), but is also located in the brush border membrane of kidney proximal tubule cells (Huls et al., 2008). Controlled feeding studies have demonstrated dose-dependent urinary lignan excretion with substantial variation in the systemic disposition and urinary excretion of enterolignans (Lampe et al., 2006); thus, the interaction of enterolactone and its conjugates with

ABCG2 may be a factor that contributes to these variations, together with interaction with the colonic environment.

Although enterolactone is an ABCG2 substrate, in the present case, no statistically significant differences between *Abcg2* knockout and wild-type mice in endogenous levels in plasma were noted. Some authors have reported no ABCG2-mediated effect in plasma systemic profile of some ABCG2 substrates, while it has proved possible to demonstrate local effects in compound transfer in the placenta or the mammary glands (Jonker et al., 2005; Zhou et al., 2008). Additional factors affecting systemic disposition, such as other transporters or metabolism, might preclude an ABCG2 effect at this level.

In addition, to characterize further the interaction of enterolactone with mouse ABCG2 and human ABCG2, the ability of this compound to modulate the accumulation of the antineoplastic agent mitoxantrone as a model ABCG2 substrate in murine *Abcg2*- and human ABCG2-expressing cell lines was tested in flow cytometry experiments. ABCG2 inhibition increased the accumulation of mitoxantrone in transduced cells, reaching $38\% \pm 2\%$ of inhibition ($200 \mu\text{M}$) (Fig. 2).

TABLE 1

Enterolactone concentration (nM) $\pm$ S.D. in plasma and milk samples from wild-type and *Abcg2*^{-/-} mice fed on standard feed (n = 5)

Enterolactone		
<i>Wild-Type</i>		
<i>Milk</i>	<i>Plasma</i>	<i>Milk/Plasma Ratio</i>
136.23	39.85	3.42
382.16	52.88	7.23
210.38	52.66	4.00
567.67	72.55	7.82
663.13	69.03	9.61
<i>Mean</i>		
391 ± 225	57 ± 13	6.4 ± 2.6
<i>Abcg2</i> ^{-/-}		
<i>Milk</i>	<i>Plasma</i>	<i>Milk/Plasma Ratio</i>
30.06	57.47	0.52
57.17	118.29	0.48
29.16	87.08	0.33
15.87	34.79	0.46
14.16	85.9	0.16
<i>Mean</i>		
$29 \pm 17^*$	77 ± 32	$0.4 \pm 0.1^*$

**P* < 0.05, significant difference between wild-type and knockout mice.

Fig. 2. Effect of the enterolignan enterolactone on the accumulation of mitoxantrone (at $10 \mu\text{M}$) in parent MDCKII cells and in their murine *Abcg2*- and human ABCG2-transduced derivatives. Cells were preincubated with or without Ko143 ($1 \mu\text{M}$) or enterolactone at the indicated concentrations. (A) Units of fluorescence, (median). (B) Percentage of inhibition of the different concentrations of enterolactone. Data are expressed as mean values $\pm$ S.D. (*n* = 3). Percentage of inhibition was related to the effect of reference inhibitor Ko143 (set at 100% inhibition of ABCG2).

These results agree with Tan et al., (2013), who described significant inhibitory activities of sinapic and ellagic acids against ABCG2-mediated transport of methotrexate, with IC₅₀ values of 117.5 and 20.7 μ M, respectively; these values are considerably less than those reported for other flavonoids. The concentrations used in the present study were physiologically significant, as the concentrations achievable in the colon lumen have been estimated to be 10–1,000 μ M (Kitts et al., 1999). Moreover, it should also be kept in mind that enterohepatic circulation and enteric recycling might lead to longer residence times, and thus to accumulation of these compounds in the body, particularly with repeated intake. Therefore, enterolactone inhibition of the ABCG2 function should be considered related to potential food-drug interactions affecting the pharmacokinetics of other ABCG2 substrates, as reported previously for dietary flavonoids (Vlaming et al., 2009; Tan et al., 2013). In addition, ABCG2 inhibitors may be useful in other fields of application, for instance in resistance reversal in chemotherapy (Allen et al., 2002; Robey et al., 2009). Further studies are needed to establish the in vivo application of this compound as an ABCG2 inhibitor.

In this study the enterolignan enterolactone was identified as an ABCG2 substrate, and its Abcg2-mediated secretion into milk was demonstrated, this might potentially affect the health benefits related to the lignan content of milk. Furthermore, the inhibitory activity of the mammalian lignan that was identified provides a basis for further investigations into possible food-drug interactions.

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5.6. ARTICLE VI

Flaxseed-enriched diets reduce danofloxacin secretion into milk in sheep by ABCG2 inhibition

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ABSTRACT

Enterolignans are phytoestrogen that exert antioxidant and cancer chemopreventive properties. Enterolactone, the main enterolignan, is produced by the rumen and intestinal microbiota from plant lignans. Flaxseed is the most common and rich dietary source of enterolignan precursors and is an acceptable supply of energy for livestock. Previous in vitro and in vivo studies have shown that enterolactone interacts with ABCG2. This transporter is involved in the secretion of many compounds including antimicrobials into milk. Danofloxacin is an antimicrobial drug for the treatment of mastitis and respiratory infections in ruminants and has been previously demonstrated to be transported into milk by ABCG2. We study here whether enterolactone inhibits ovine ABCG2-mediated transport of danofloxacin in vitro and in vivo. We used enterolactone in MDCK-II cells overexpressing the ovine ABCG2 to inhibit the in vitro transport of danofloxacin. To study in vivo whether enterolactone reduces the amount of danofloxacin secreted into milk, 3 different groups of sheep were fed with 3 different diets containing variable amounts of flaxseed for 2 weeks. Danofloxacin was administered (1.25 mg/kg) to sheep, and plasma and milk samples were analyzed by HPLC-fluorescence detection. No differences were noted in plasma pharmacokinetics between the 3 groups of animals. In contrast, milk danofloxacin pharmacokinetics showed that $AUC_{0-\infty}$, C_{max} and $AUC_{0-\infty}$ milk-to-plasma ratios were reduced by 25-30% in animals fed with flaxseed-enriched diets. These results demonstrate that flaxseed-enriched diets reduce the expected levels in milk of an ABCG2 substrate, such as danofloxacin, thus highlighting the relevance of flaxseed-supplemented diets not only for ruminant milk quality but for the control of potential drug residues.

Keywords: ABCG2, lactation, danofloxacin, mammary

25 **Introduction**

26 Lignans are polyphenolic substances that can be found in varying amounts in almost all
27 plants. Flaxseed (*Linum usitatissimum*) is the richest source of plant lignans with
28 elevated quantities of the enterolignan precursor secoisolariciresinol diglucoside (SDG)
29 (Axelson et al., 1982). Metabolism of SDG by ruminal and intestinal microbiota leads
30 to the formation of mammalian enterolignans, i.e. enterodiols and in particular
31 enterolactone (Schogor et al., 2013). Conjugation of enterolignans, mainly with sulfate
32 and glucuronic acid, occurs in the digestive tract and liver (Gagnon et al., 2009; Njastad
33 et al., 2014). Enterolactone has been reported to exert interesting human health benefits
34 with respect to menopausal symptoms, breast and prostate cancers, cardiovascular
35 diseases, osteoporosis and diabetes (Adlercreutz, 2002). Besides its beneficial effects on
36 health, enterolactone has been shown to have interesting properties for the veterinary
37 industry since it improves survival of cow embryos, and so has a positive effect on
38 cattle fertility (Petit and Gagnon 2009).

39 Recently, our group reported that accumulation of enterolactone in milk was mediated
40 by the ABCG2 transporter (Miguel et al., 2014). ABCG2 is a protein apically expressed
41 in the plasma membrane of cells, widely studied for its capacity to extrude several drugs
42 and natural compounds out of cells. It is prominently expressed in hepatocytes and
43 enterocytes among other cell types and is directly implicated in molecule absorption and
44 bioavailability (Jani et al., 2014). This transporter was shown to be overexpressed
45 during lactation in the mammary gland epithelial alveolar cells (Farke et al., 2008).
46 (Jonker et al., 2005) demonstrated that ABCG2 was responsible for concentrating
47 carcinogenic agents in milk, and (van Herwaarden et al., 2007) studied thereafter its
48 implication in the secretion of riboflavin (vitamin B2), which also suggested a relevant
49 interaction with natural compounds. Previous studies have shown that ABCG2-

mediated drug-drug interactions can alter drug pharmacokinetics (Nigam, 2015) and inhibit drug transport into milk (Real et al., 2011b). However, ABCG2 inhibition by xenobiotics with additional adverse side effects may not be the optimal scenario in some cases. The use of natural compounds for this purpose could have additional beneficial effects. Therefore, some previous studies have dealt with the effect of dietary compounds on drug pharmacokinetics and secretion into milk mediated by ABCG2. For example, isoflavone derivatives interact with ABCG2 in the mammary gland and thus, soy-enriched diets (with high amounts of isoflavones) can reduce milk secretion of fluoroquinolones (Pulido et al., 2006).

In this regard, the objective of the present study was to determine the capacity of enterolactone, the main enterolignan, to inhibit *in vitro* and *in vivo* ABCG2-mediated transport of the antimicrobial danofloxacin. We studied therefore the effect of enterolactone on drug transepithelial transport assays in cells overexpressing ovine ABCG2, and we analyzed the effect of flaxseed-enriched diets on plasma and milk disposition of danofloxacin in sheep.

Material and Methods

Reagents and Drugs

Enterolactone and enterodiol were obtained from Sigma-Aldrich (St. Louis, MO) and danofloxacin (Advocin 2.5%) from Pfizer (Cedex, France). All other chemicals were analytical grade and available from commercial sources.

Cell Cultures

Madin-Darby canine kidney epithelial cell (MDCKII) parental cells were provided by Dr A.H. Schinkel (Netherlands Cancer Institute). Stably-transduced MDCKII cells with ovine variant of ABCG2 were previously generated and characterized by our group (Gonzalez-Lobato et al., 2014). The cells were cultured in Dulbecco's modified Eagle's medium containing Glutamax (Thermo Fisher Scientific, Madrid, Spain) and supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL) and 10% (vol/vol) fetal calf serum (Thermo Fisher Scientific) at 37 °C in the presence of 5% CO₂. The cells were treated with trypsin every 3–4 days for sub-culturing.

Transport Studies

Transepithelial transport assays were carried out as previously described with minor modifications (Pavek et al., 2005; Real et al., 2011b). Cells were seeded on microporous polycarbonate membrane filters (3.0 µm pore size, 24 mm diameter; Transwell, Costar, Corning, NY) at a density of 1.0×10^6 cells per well. Cells were grown for 3 days, and the medium was replaced every day. Transepithelial resistance was measured in each well using a Millicell ERS ohmmeter (Millipore Corporation, Billerica, MA). Wells registering a resistance of 200 X or greater, after correcting for the resistance obtained in blank control wells, were used in the transport experiments. The measurement was repeated at the end of the experiment to check the tightness of the monolayer. Before

the start of the experiment, medium on both sides of the monolayer was replaced with 2 mL of Optimem medium (Life Technologies), without serum, either without or with the inhibitor (50 or 100 μ M enterolactone). After 2 h incubation, the experiment was started (t = 0 h) by replacing the medium in either the apical or basolateral compartment with fresh Optimem medium containing 10 μ M danofloxacin, with or without the inhibitor. Aliquots of 100 μ L were taken from the opposite compartment at t = 2 and 4 h, and stored at -20 °C until high performance liquid chromatography (HPLC) analysis. The appearance of danofloxacin in the acceptor compartment was presented as the fraction of total danofloxacin added to the donor compartment at the beginning of the experiment. Active transport across MDCK-II monolayers was expressed by the relative transport ratio, defined as the apically directed transport percentage divided by the basolaterally directed translocation percentage, after 4 h.

Pharmacokinetic Studies

The experiments were performed at the Experimental Farm of the University of Leon. Experimental procedures were approved by the Research Committee of Animal Use at the University of Leon (Spain). Animals were housed and handled according to institutional guidelines complying with European legislation (2010/63/EU). Eighteen lactating Assaf sheep (3–4 months in lactation and weighing 66–105 kg) were used. The sheep were milked twice daily. The animals were parasite-free, and drinking water was available ad libitum. The experimental design was performed with animals divided into three groups of six animals each. One group of sheep was fed with a standard diet, a second group was fed with a lignan-enriched diet containing 10% whole flaxseed (Nutricor SL, Leon, Spain) for 2 weeks, and a third group was fed with a lignan-enriched diet containing 15% whole flaxseed (Nutricor SL) also for 2 weeks (diet composition is shown in Table 1). After 2 weeks, all groups received a single dose (1.25

mg/kg, i.m.) of danofloxacin (Advocin 2.5 %). Blood samples were collected from the jugular vein at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after danofloxacin administration. Plasma was separated by centrifugation at 1000 x g for 15 min and stored at -20 °C until analysis. Milk samples were collected after complete milking of the gland, before each treatment, and at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h after danofloxacin administration. Samples were stored at -20 °C until analysis.

UPLC-QTOF-MS Analysis of Enterolactone and Related Compounds

Plasma and milk samples (100 µL) were mixed with sodium acetate buffer and treated with hydrochloric acid in methanol, following the method of Bolca et al. (2010), redissolved in methanol, and filtered (0.22 µm) prior to injection into the liquid chromatography–mass spectrometry system.

Analyses were developed using an Agilent 1290 Infinity LC system coupled to a 6550 Accurate-Mass QTOF (Agilent Technologies, Waldbronn, Germany) and using an electrospray interface (Jet Stream Technology). Analytical conditions were the same as previously described with some modifications (Miguel et al., 2014). Briefly, 5 µL of sample was injected on a reverse phase Poroshell 120 EC-C18 column (3 x 100 mm, 2.7 µm) (Agilent Technologies) operating at 30 °C. Water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as mobile phases at a flow rate of 0.4 mL/min and the following gradient: 5-25% B at 0-10min, 25-40% B at 10-20 min; 40-90% B at 20-24 min; 90%-5% at 25-26 min and the column re-equilibrated for 4 min. Spectra were acquired in the range 100-1100 *m/z* in negative mode and the conditions described above.

Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00 Agilent Technologies). A target screening strategy was applied to plasma and

milk samples for the qualitative screening of possible enterolignan-related metabolites. A list of target compounds (60 possible metabolites) after MS full-acquisition was performed. Screening was based on mass filtering at the exact mass of the compound investigated using narrow mass extraction windows (0.01 m/z). Identification was possible from the valuable information given in QTOF-MS acquisition mode that provides possible molecular formulae for the compounds based on the accurate mass and isotopic pattern. Additionally, targeted MS/MS experiment offered fragmentation information providing more confidence in the compound identification process. When possible, direct comparison with authentic standards was performed. Quantification of enterolactone and enterodiol was determined by interpolation in the calibration curve obtained with their own available standards. These metabolites were quantified in MS by peak area integration of their extracted ion chromatograms.

HPLC Analysis of Danofloxacin

The conditions for HPLC analysis of danofloxacin concentrations in plasma and milk were modified according to previously published methods (Cho et al., 2010). Difloxacin at 0.1 µg/mL for the plasma sample extraction and 0.01 µg/mL for the milk sample extraction were used as internal standards. To each 100 µL aliquot of sample 600 µL of chloroform was added. Samples were shaken and the organic and water phases were separated by centrifugation at 5,000 x g for 6 min and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 µL of methanol and injected into the HPLC system consisting of a Waters 600 pump, a Waters 717 plus autosampler, and a Waters 486 fluorescence detector (Waters Corporation, Milford, MA). Separation of the samples was achieved with a C₁₈ reversed-phase column (Mediterranea Sea 18 5 µm 250x0.46 mm; Teknokroma, Barcelona, Spain). Mobile phase consisted of 25 mM orthophosphoric acid supplemented with 0.1% triethylamine (pH 3.0): acetonitrile

(80:20 vol/vol) at a flow rate of 1.6 mL/min. Sample detection was performed by fluorescence detection at 338 nm (excitation) and 425 nm (emission). Integration was carried out using MILLENNIUM 32 software (Waters).

Standard samples in the appropriate drug-free matrix were prepared, yielding a concentration range from 0.078 to 10 µg/mL for transport samples. For pharmacokinetic analyses standards in plasma ranged from 0.0019 to 0.5 µg/mL and in milk from 0.019 to 5 µg/mL. Correlation coefficients (r) for danofloxacin ranged between 0.998 and 0.984 for the calibration curves in plasma and milk curves respectively. LOQs were 1.9 ng/mL for sheep plasma and 19 ng/mL for sheep milk.

Pharmacokinetic Calculations and Statistical Analyses

Peak concentration ($C_{\max}$) and time–peak concentrations ($T_{\max}$) were read from the plotted concentration–time curve for each animal. The area under concentration–time curves (AUC) from time zero to infinity was calculated using the trapezoidal method ($AUC_{0-\infty}$). Mean residence time (MRT) was calculated by the linear trapezoidal rule without extrapolation to infinity, using the formula: $MRT = AUMC/AUC$; where AUMC is the mean area under the momentum curve. The rate constant (λ_z), associated with the terminal elimination phase, was estimated by means of linear regression of the terminal phase of the log concentration versus time profile, and was used to calculate the corresponding elimination half-life ($T_{1/2el}$). These calculations were made with a computer program (PK solution 2.0, Summit Research Services, Ashland, OH) and determined by non-compartmental analyses. Results are presented as the mean $\pm$ standard error of the mean (SEM). Statistical evaluation of data was carried out by analysis of variance (ANOVA) followed by the Newman–Keuls test. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Inhibition of Danofloxacin Transport by Enterolactone in MDCK-II Cell Line Transduced with the oABCG2

To demonstrate the capacity of enterolactone to inhibit the ABCG2 transporter, MDCK-II cells stably overexpressing the ovine variant of the transporter were used. This model was recently established by our group (Gonzalez-Lobato et al., 2014).

To test our hypothesis we checked the effect of two different enterolactone concentrations (50 and 100 μ M) on danofloxacin (10 μ M) transport that resulted in the partial and complete inhibition, respectively, of the drug transport mediated by ABCG2 (Figure 1). Parental MDCK-II showed a relative transport ratio of ≈ 1.1 , in the absence and presence of both concentrations of enterolactone, indicating that equal amounts of drug are translocated from the apical to the basal compartment and viceversa with all the treatments. MDCK-II cells overexpressing the ovine variant of the ABCG2 transporter showed a relative transport ratio for danofloxacin of 9.22 in the absence of enterolactone, which indicated a preferential transport of danofloxacin to the apical side, thus confirming that danofloxacin is an ovine ABCG2 substrate as previously reported by Gonzalez-Lobato et al. (2014). Enterolactone 50 μ M partially inhibited the ovine ABCG2-mediated transport of danofloxacin reducing the relative transport ratio to 2.48. When a concentration of enterolactone of 100 μ M was used, the ovine ABCG2-mediated transport was inhibited almost completely and showed a transport ratio of 1.3, similar to the transport ratio of the parental MDCK-II cell line (Figure 1).

Our results show, therefore, that enterolactone is an inhibitor of ovine ABCG2-mediated in vitro transport of danofloxacin.

Plasma Pharmacokinetics and Danofloxacin Secretion into Milk in Sheep Fed with Variable Amounts of Whole Flaxseed

To further demonstrate the in vivo ABCG2 inhibitory role of enterolactone, the effect of flaxseed-enriched diets (containing high levels of the enterolactone precursor SDG) on pharmacokinetics and secretion into milk of danofloxacin was studied in sheep. Eighteen sheep were separated into three different sets: a control diet without flaxseed and the second and the third groups which received a diet with 10% and 15% flaxseed, respectively. After 2 weeks with the experimental diets, levels of enterolactone and related compounds were analysed in plasma and milk, prior to danofloxacin administration. All the groups received a single dose of danofloxacin 1.25 mg/kg and plasma and milk samples were analysed for danofloxacin concentrations.

Enterolignan (enterolactone and enterodiol) levels increased in plasma and milk from animals fed with the flaxseed-enriched diets (Figure 2). The enterolactone precursor enterodiol was present in plasma and milk only in the groups fed with flaxseed. Enterolactone-glucuronide, enterodiol-glucuronide, enterolactone-sulfate and enterodiol-sulfate were only detected in plasma of the group with 15% flaxseed (data not shown). Enterolactone and enterodiol conjugates were not detected in milk.

Plasma analyses revealed that the different diets had no effect on plasma distribution and pharmacokinetic parameters of danofloxacin (Figure 3, Table 2). However, milk concentrations of danofloxacin and its pharmacokinetic parameters significantly differed between the groups. According to Figure 4, flaxseed-enriched diets significantly reduced the amount of danofloxacin secreted into milk. $AUC_{0-\infty}$ values were reduced by approximately 25-30% (Table 3). C_{max} values diminished by almost 40% in the group fed with 15% flaxseed (2.95 ± 0.30 μ g/mL control group vs. $1.84 \pm$

0.18 $\mu\text{g/mL}$ 15% flaxseed group, $P < 0.05$). $\text{AUC}_{0-\infty}$ milk/plasma ratios also were significantly reduced in the groups fed with flaxseed-enriched diets (11.12 ± 0.80 in the control group vs. 7.86 ± 0.84 and 7.64 ± 0.86 in the 10% and 15% flaxseed groups respectively, $P < 0.05$). MRT and $T_{1/2\text{ el}}$ parameters did not reach significant differences. The groups fed with 10% and 15% flaxseed showed similar results and no statistical differences were obtained for any of the parameters analyzed.

These results demonstrate that flaxseed-enriched diets reduce the levels of danofloxacin in milk, according to the inhibitory role of enterolactone on the transport of this antimicrobial.

Discussion

The role of natural products present in the diet of ruminants as modulators of secretion into milk of drugs is a new line of veterinary research which is very useful for the control of drug residues in milk and for the design of accurate therapies for mastitis treatment. The effect of soy-enriched diets containing isoflavones has been reported to limit drug secretion into sheep's milk by modulating ABCG2 activity (Perez et al., 2009, 2013). We report here that flaxseed-enriched diets can be included in the group of dietary modulators for the secretion of antimicrobials into milk and we identify the enterolignan enterolactone as a critical metabolite involved in this modulation.

Previous studies have shown enterolactone to be an excellent substrate of different variants of the ABCG2 transporter (Miguel et al., 2014), suggesting that enterolactone could act as a competitor against other ABCG2 substrates. Furthermore, these authors demonstrated that enterolactone efficiently inhibited ABCG2-mediated mitoxantrone transport using cells overexpressing murine and human ABCG2. In the present paper, cells overexpressing ovine ABCG2 were used, showing that enterolactone inhibits the ovine ABCG2-mediated transepithelial transport of danofloxacin (Figure 1), and thus demonstrating that enterolactone is an efficient inhibitor of ABCG2-mediated danofloxacin transport.

To study the potential inhibition of ovine ABCG2 by enterolactone in vivo, an experimental design using flaxseed-supplemented diet was developed. Flaxseed is the main source of enterolignans generally used in farms. Whole flaxseed supplementation in diet for cows is generally considered as an acceptable source of protein and energy. A source of SDG, an enterolactone precursor, promotes the biotransformation of this precursor to other enterolignans and the concentration of enterolactone in milk.

Increasing amounts of flax in meal has been directly related with a higher amount of enterolactone in plasma, urine and milk in dairy cattle (Gagnon et al., 2009; Cortés et al., 2013). However, to the best of our knowledge, this is the first report of levels of enterolactone and related compounds in plasma and milk after flaxseed supplementation in sheep (Figure 2). In this paper, we demonstrate for the first time in sheep a direct correlation between the amount of whole flaxseed added to the diet and the amount of enterolactone found in plasma and milk. Our data show increased levels of enterolactone in sheep's plasma and milk after 2 weeks of flaxseed supplementation (Figure 2). Enterolactone is mainly produced in the rumen and *Prevotella* spp. has proved to play a central role in SDG biotransformation and intestinal absorption in cattle (Schogor et al., 2014). Nevertheless, it is known that a number of as yet unidentified precursors can also lead to formation of mammalian enterolignans, at least in cattle (Njastad et al., 2014).

These animals with variable levels of enterolactone in plasma and milk (Figure 2) were administered danofloxacin at 1.25 mg/kg. Our analysis of danofloxacin plasma levels were in accordance with previous reports in sheep (Aliabadi et al., 2003; Barrera et al., 2013). Our results reveal no effect on danofloxacin plasma levels and pharmacokinetic parameters with the diets containing different amounts of flaxseed (Figure 3, Table 2). Danofloxacin studies in *Abcg2* (-/-) mice did not show any alteration in plasma levels of danofloxacin (Real et al., 2011a), probably due to the fact that several other transporters and physiological processes are acting on overall plasma availability of danofloxacin.

With regard to results in milk, levels of danofloxacin in the control group are in agreement with those reported by Barrera et al. (2013) and Perez et al. (2013). In sheep fed with flaxseed diet, secretion of danofloxacin into milk was reduced by 25-30% (Figure 4, Table 3), which might indicate a competition process between microbial-diet

312 derived enterolactone and danofloxacin in a similar way to the in vitro experiments. The
313 increase of flaxseed in the diet (from 10 to 15%) did not produce a proportional
314 reduction in the amount of danofloxacin in milk. It is likely that the high concentrations
315 of enterolactone obtained in plasma in the animals fed with flaxseed-enriched diet
316 (Figure 2) produced a saturation of the ABCG2-mediated transport of enterolactone
317 (and probably its related compounds) in the milk. The similar milk concentrations of
318 enterolactone and its precursor enterodiol (and the absence of their conjugates in milk)
319 reached with the two flaxseed-enriched diets (10 and 15%), despite the different plasma
320 concentrations, reinforce the saturation hypothesis. Phytoestrogen concentration in milk
321 has been suggested to be modulated through diet intake although transfer capacity to
322 milk appears to be limited by saturation (Njastad et al., 2014). Therefore, the similar
323 amounts of enterolactone in milk with the two flaxseed-enriched diets (10 and 15%)
324 could be the cause of the similar effect on danofloxacin secretion into milk observed
325 with the two diets.

326 It cannot be ruled out that other enterolignan-derivatives, such as precursors (enterodiol)
327 or enterolactone derivatives, could have an additional effect on ABCG2-mediated
328 danofloxacin secretion into milk. Our data reveal the presence of conjugates of
329 enterolactone and enterodiol only in plasma (not in milk) of the group fed with 15%
330 flaxseed (data not shown). Moreover, the similar effect on danofloxacin secretion into
331 milk observed in the groups fed with 10% and 15% flaxseed suggests that these
332 conjugates probably do not have an additional effect on ABCG2-mediated transport of
333 danofloxacin. Further studies are needed to test the interaction of other enterolactone-
334 related compounds with ABCG2.

335 ABCG2 inhibition is an interesting issue due to the multiple facets that this drug
336 transporter has, including drug secretion into milk. The beneficial properties of

enterolactone and its efficiency in ABCG2 inhibition open up the possibility of using it as an inhibitor of the transporter in contrast to other efficient ABCG2 inhibitors of a xenobiotic nature that present a number of toxic side effects (Mao and Unadkat2015b). A combination of dietary enterolignan precursors with other phytoestrogens such as isoflavones could have a synergistic effect on ABCG2-mediated transport, potentially affecting drug secretion into milk. Red and white clover silages have been demonstrated to increase levels of isoflavones and enterolignans in milk, respectively, thus concentrating phytoestrogens in milk. The increased content of phytoestrogens in milk might impart health benefits to milk (Steinsham et al., 2008).

Our results highlight the relevance of flaxseed diets supplied to livestock that modulate not only milk nutrient composition but also the amount of potential residues that can eventually be found in milk.

CONCLUSIONS

In this work we demonstrate that ABCG2 danofloxacin transport is inhibited by flax-enriched diets and we identify the dietary-derived metabolite enterolactone as an inhibitor of ovine ABCG2. This finding presents the possibility of reducing drug potential residues in ruminants' milk by supplying diets with flaxseed and may help to handle milk pollutants that interact with ABCG2.

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Table 1

Composition of the diets

<i>Ingredients (%)</i>	Control	Flaxseed 10%	Flaxseed 15%
Dehydrated alfalfa hay	40.0	40	40.0
Whole flaxseed	0.0	10	15.0
Corn grain	18.6	18.6	15.0
Rape seed	13.7	11	10.0
Barley grain	6.3	5	5.6
Beet pulp	6.0	3	3.0
Oat grain	5.0	3	5.0
Cotton seed	5.0	4	1.0
Molasses	4.0	4	4.0
Vitamin and mineral supplements	1.4	1.4	1.4
<i>Analysis</i>			
Dry matter (%)	90.90	91.02	91.08
Neutral detergent fibre (%)	30.79	29.33	29.04
Crude protein (%)	18.06	18.05	18.05
Energy (Mj/kg)	11.89	12.14	12.21

Table 2

Plasma pharmacokinetic parameters (means $\pm$ SEM) of danofloxacin after i.m. administration at a dosage of 1.25 mg/kg in control diet group (n = 6), 10% flaxseed enriched diet group (n = 6) and 15% flaxseed enriched diet group (n = 6).

Parameter ¹	Control	Flaxseed 10%	Flaxseed 15%
AUC_{0-∞} (μg·h/mL)	1.75 $\pm$ 0.15	1.93 $\pm$ 0.10	1.81 $\pm$ 0.06
C_{max} (μg/mL)	0.31 $\pm$ 0.01	0.31 $\pm$ 0.02	0.30 $\pm$ 0.01
MRT (h)	4.22 $\pm$ 0.29	4.77 $\pm$ 0.19	4.95 $\pm$ 0.15
T_{max} (h)	1.50 $\pm$ 0.20	1.50 $\pm$ 0.20	1.16 $\pm$ 0.15
T_{1/2 el} (h)	2.92 $\pm$ 0.20	3.31 $\pm$ 0.13	3.43 $\pm$ 0.10

¹AUC, area under the concentration curve; C_{max}, maximum concentration; MRT, mean residence time; T_{1/2 el}, elimination half-life; T_{max}, time to peak concentration.

Table 3

Milk pharmacokinetic parameters (means $\pm$ SEM) of danofloxacin after i.m. administration at a dosage of 1.25 mg/kg in control diet group (n = 6), 10% flaxseed enriched diet group (n = 6) and 15% flaxseed enriched diet group (n = 6).

Parameter ¹	Control	Flaxseed 10%	Flaxseed 15%
AUC_{0-∞} (μg·h/mL)	19.44 $\pm$ 2.09 ^a	14.86 $\pm$ 0.76 ^b	13.68 $\pm$ 1.27 ^b
C_{max} (μg/mL)	2.95 $\pm$ 0.30 ^a	2.20 $\pm$ 0.14 ^b	1.84 $\pm$ 0.18 ^b
MRT (h)	5.35 $\pm$ 0.35	5.25 $\pm$ 0.14	5.54 $\pm$ 0.11
T_{max} (h)	2.66 $\pm$ 0.38	3.00 $\pm$ 0.40	2.66 $\pm$ 0.38
T_{1/2 el} (h)	3.71 $\pm$ 0.24	3.64 $\pm$ 0.10	3.84 $\pm$ 0.08
AUC Milk/plasma	11.12 $\pm$ 0.80 ^a	7.86 $\pm$ 0.84 ^b	7.64 $\pm$ 0.86 ^b

^{a,b} Means within a row with different superscripts differ ($P < 0.05$).

¹AUC, area under the concentration curve; C_{max}, maximum concentration; MRT, mean residence time; T_{1/2 el}, elimination half-life; T_{max}, time to peak concentration.

Figure 1. Transepithelial transport of danofloxacin (10 μ M) in parental MDCKII and its ovine ABCG2-transduced derivatives in the presence or absence of enterolactone 50 and 100 μ M. Results are the means; error bars (sometimes smaller than the symbols) indicate the SEM ($n \geq 3$). Translocation from the basolateral to the apical compartment ($\square$); translocation from the apical to the basolateral compartment ($\blacksquare$). r represents the relative transport ratio (i.e., the apically directed translocation divided by the basolaterally directed translocation) at 4 h.

Figure 2. Enterodiol and enterolactone concentrations (nM) in plasma and milk in animals fed with flaxseed-enriched diets for two weeks in control diet group ($n = 6$), 10% flaxseed-enriched diet group ($n = 6$) and 15% flaxseed-enriched diet group ($n = 6$). The results are presented as means; error bars indicate SEM.

Figure 3. Plasma concentrations of danofloxacin after its i.m. administration at a dosage of 1.25 mg/kg to sheep fed with control diet (n=6) (■), 10% flaxseed-enriched diet (n=6) (Δ) and 15% flaxseed-enriched diet (n=6) (◆). Plasma samples were collected at several points over 24 h. The results are presented as means; error bars indicate SEM.

Figure 4. Milk concentrations of danofloxacin after its i.m. administration at a dosage of 1.25 mg/kg to sheep fed with control diet (n=6) (■), 10% flaxseed-enriched diet (n=6) (Δ) and 15% flaxseed-enriched diet (n=6) (◆). Milk samples were collected at several points over 24 h. The results are presented as means; error bars indicate SEM.

6. DISCUSIÓN

6.1. Caracterización *in vivo* del polimorfismo Y581S del ABCG2 bovino

6.1.1. Efecto del polimorfismo sobre la transferencia de fármacos a leche

La transferencia de fármacos a leche se puede considerar un problema en las explotaciones ganaderas para la producción intensiva de productos lácteos. La aparición residual de fármacos en leche puede generar el desarrollo de resistencias bacterianas, alergias e hipersensibilidad en los consumidores de productos lácteos. Sin embargo, la transferencia de fármacos a la leche también permite la llegada de determinados principios activos a la glándula mamaria, favoreciendo el tratamiento de enfermedades como la mastitis. Por ello, la determinación de aquellos factores implicados en el paso de fármacos a la leche es un área de gran relevancia para la salud pública, la industria láctea y la medicina veterinaria. Tras el descubrimiento del polimorfismo Y581S del transportador ABCG2 bovino realizado por Cohen-Zinder et al., (2005) nuestro grupo demostró una mayor actividad de la variante S581 frente a la variante Y581 utilizando el modelo celular MDCK-II transducido de manera estable con las dos variantes del transportador bovino (Real et al., 2011b). Sin embargo, hasta el momento ningún estudio *in vivo* avalaba estos resultados obtenidos en el laboratorio. Por ello, en esta tesis doctoral se ha estudiado en profundidad el efecto de dicho polimorfismo en la disponibilidad plasmática y la secreción a leche de diferentes fármacos ampliamente utilizados en la cabaña ganadera.

El danofloxacino es un antimicrobiano del grupo de las fluoroquinolonas ampliamente utilizado para tratar infecciones del tracto respiratorio, digestivo y de la glándula mamaria (Schrickx and Fink-Gremmels, 2007; Poutrel et al., 2008). Los residuos en leche derivados del tratamiento con fluoroquinolonas son de especial interés debido a que son muy resistentes a los tratamientos térmicos, por lo cual podrían persistir en los alimentos tras los procesamientos industriales que los habilitan para el consumo (Roca et al., 2010). Los estudios previos de Real et al., (2011a) apuntaban al danofloxacino como un excelente sustrato *in vitro* del transportador

bovino ABCG2 que además se transportaba de manera diferente entre las dos variantes. Por ello, se optó por un primer ensayo en el cual el danofloxacino fue administrado a 6 vacas carentes del polimorfismo (Y/Y) y 6 vacas heterocigotas para el polimorfismo Y581S (Y/S) a una dosis de 1,25 mg/kg y se analizaron sus niveles en plasma y leche (artículo I). Los resultados obtenidos en plasma fueron muy similares a los obtenidos por Shem-Tov et al., (1998) y Shojaee Aliabadi and Lees, (2003), no observándose diferencias en las AUC plasmáticas entre los dos genotipos ($1,59 \pm 0,29$ $\mu\text{g}\cdot\text{h}/\text{ml}$ en vacas Y/Y frente $1,44 \pm 0,12$ $\mu\text{g}\cdot\text{h}/\text{ml}$ en vacas Y/S (Figura 1, tabla 1, artículo I)). Aunque es habitual que ABCG2 tenga la capacidad de afectar los niveles plasmáticos de sus sustratos (Kim et al., 2007; Keskitalo et al., 2009), existen estudios que han demostrado que para ciertos sustratos el perfil plasmático no se ve afectado, incluso en ausencia del transportador como sucede en los ratones *Abcg2*^(-/-) (Vlaming et al., 2009). Así, estudios previos de nuestro grupo de investigación en modelos murinos y ovinos han corroborado que el transportador ABCG2 no afecta las concentraciones plasmáticas de danofloxacino (Real et al., 2011a; Barrera et al., 2013). Este no es un hecho aislado ya que otros polimorfismos con relevancia clínica como el polimorfismo humano Q141K del transportador ABCG2 no tienen efecto alguno sobre la disposición plasmática de ciertos sustratos (Kim et al., 2007; Adkison et al., 2008; Keskitalo et al., 2009). Esto se puede deber a la existencia de otros transportadores implicados en la disponibilidad plasmática de estas moléculas de manera que se amortigua un potencial efecto producido por ABCG2. En cualquier caso la ausencia de efecto del polimorfismo sobre la concentración plasmática de danofloxacino beneficia el tratamiento de infecciones sistémicas ya que una alteración de los niveles del antimicrobiano en función del polimorfismo podría comprometer el éxito de la terapia debido a que las fluoroquinolonas tienen un efecto dosis-dependiente.

En lo que respecta a la leche, los resultados obtenidos están en concordancia con los obtenidos por Shem-Tov et al., (1998) aunque sólo para las vacas Y/Y. Los niveles de danofloxacino encontrados en las vacas Y/S son significativamente mayores que los detectados en las vacas Y/Y a las 5 y 11 horas (Figura 2, artículo I). Los ratios de las AUCs leche/plasma indican que las vacas Y/S tienen la capacidad de transferir más del doble de fármaco del plasma a la leche ($4,09 \pm 1,07$ en vacas Y/Y frente $8,81 \pm 3,69$ en vacas Y/S). Además los parámetros de eliminación, tiempo de residencia medio (MRT) y vida media de eliminación ($T_{1/2}$) fueron significativamente menores en las vacas Y/S (Tabla 1, artículo I). Las fluoroquinolonas son rápidamente eliminadas del organismo (Shem-Tov et al., 1998; Vethanayagam et al., 2005). El periodo de supresión para esta dosis es de solamente 48 horas, lo cual dificulta el estudio del efecto del polimorfismo sobre el MRT o la vida media. Serían necesarios estudios farmacocinéticos con otros fármacos sustrato con un mayor tiempo de residencia medio para poder evaluar correctamente el efecto del polimorfismo sobre un parámetro tan crítico para la industria láctea como es el MRT. En cualquier caso los espectaculares resultados obtenidos en las vacas heterocigotas en lo que respecta a su mayor capacidad para secretar este antimicrobiano a la leche están en concordancia con los estudios previos que se habían realizado *in vitro* por nuestro grupo, validando por otra parte la idoneidad y valor predictivo de nuestros modelos *in vitro*.

Dados los resultados positivos obtenidos con este sustrato decidimos realizar un nuevo experimento (artículo II) con una formulación más novedosa del danofloxacino; el Advocin 160® (6 mg/kg), que permite alcanzar una mayor concentración plasmática de dicho fármaco e incrementar la seguridad y éxito del tratamiento además de estar recomendado para el tratamiento de mastitis (artículo II). Dado el papel de ABCG2 en la concentración de fármacos en el entorno de la glándula mamaria las implicaciones del efecto del polimorfismo Y581S resultan especialmente interesantes en este caso.

Los resultados obtenidos a partir del análisis de los niveles plasmáticos en este nuevo estudio (Figura 2, tabla 1, artículo II) son similares a los obtenidos por Mestorino et al., (2009). En este caso tampoco se obtuvieron diferencias en el plasma entre los dos grupos de vacas aunque si se observó una tendencia en los niveles plasmáticos, con AUCs sensiblemente menores en las vacas Y/S ($7,88 \pm 2,21 \mu\text{g}\cdot\text{h/ml}$) en comparación con las vacas Y/Y ($10,83 \pm 3,38 \mu\text{g}\cdot\text{h/ml}$) aunque dichas diferencias no alcanzaron la significación ($p=0,16$) (Figura 2, tabla 1, artículo II). En lo que respecta a las parámetros farmacocinéticos de danofloxacino en leche, los obtenidos en nuestro estudio son entre dos y tres veces superiores a los obtenidos en otros trabajos (Mestorino et al., 2009), lo cual puede atribuirse a diferencias en el diseño experimental, así como a las condiciones fisiológicas de los animales cuyo peso y producción láctea fueron muy inferiores a los utilizados en nuestro estudio. Además la composición de la dieta es otro factor que puede afectar drásticamente a la disponibilidad de sustratos de ABCG2 como sucede con los flavonoides naturales. Actualmente se sabe que una dieta enriquecida en isoflavonas puede afectar a la secreción a leche de sustratos de ABCG2 (Pulido et al., 2006; Perez et al., 2013).

En lo que respecta a las diferencias entre los polimorfismos nuestros análisis demostraron que las AUCs para la leche eran un 50% mayores para las vacas Y/S en comparación con las vacas Y/Y (Figura 3, artículo II). Este efecto en términos absolutos es más moderado que el obtenido al utilizar una dosis $1,25 \text{ mg/kg}$, lo cual ilustra la importancia y la necesidad de realizar estudios específicos en función de la dosis a la que se administran los compuestos ya que la respuesta puede variar. Sin embargo, como consecuencia de los menores niveles de fármaco en el plasma de los individuos Y/S, si atendemos al ratio AUC leche/plasma observamos que la capacidad para transferir el danofloxacino a leche sigue siendo aproximadamente del doble en las vacas polimórficas ($3,42 \pm 1,32$ en vacas Y/Y frente $7,02 \pm 3,12$ en vacas Y/S (Tabla I, artículo II)). Con estos resultados queda corroborado que el polimorfismo

Y581S incrementa la secreción de danofloxacin a leche con implicaciones especialmente relevantes para el tratamiento de mastitis por las amplias diferencias que se observan en función del genotipo.

Otro fármaco ampliamente administrado en bóvidos es el enrofloxacin. La administración de este fármaco resulta en su rápida metabolización a ciprofloxacino, su principal metabolito activo. Previo al estudio del comportamiento farmacocinético del enrofloxacin y su metabolito activo ciprofloxacino en relación al polimorfismo bovino Y581S se realizó un estudio *in vitro* para evaluar el potencial impacto de este polimorfismo *in vivo*. El enrofloxacin había sido previamente testado por Real et al. (2011b), quien demostró que las células que sobreexpresaban el transportador con la variante S581 eran capaces de transportar el enrofloxacin de manera mucho más eficiente que las células transducidas con la variante Y581 al igual que sucede para el antimicrobiano danofloxacin. Para completar el estudio *in vitro*, se llevó a cabo un estudio de transporte transepitelial con el ciprofloxacino, para determinar si también interactuaba de manera diferencial con las dos variantes, al igual que su precursor enrofloxacin (artículo III). Los resultados obtenidos demostraron que el ciprofloxacino era sustrato del transportador bovino y que, al igual que el compuesto parental enrofloxacin, la variante S581 transportaba de manera más eficiente este compuesto que la variante Y581 (Figura 2, artículo III).

Para evaluar *in vivo* si el transportador ABCG2 está involucrado en la secreción de enrofloxacin a leche, se llevó a cabo un experimento de lactación con ratones *wild-type* y Abcg2^(-/-) demostrando que Abcg2 está implicado en la secreción a leche de este fármaco ya que la secreción de enrofloxacin en los ratones Abcg2^(-/-) fue significativamente menor que en los *wild-type* ($2,23 \pm 0,20$ µg/ml en ratones *wild-type* frente $1,74 \pm 0,26$ µg/ml en los ratones Abcg2^(-/-) (Figura 1, artículo III)). El papel de Abcg2 en la secreción del metabolito ciprofloxacino ya había sido estudiado por Merino et al. (2006), revelando que el transportador Abcg2 concentraba en leche dicho

compuesto. Estos estudios subrayan la fuerte interacción e influencia del transportador ABCG2 en la secreción a leche de enrofloxacin y ciprofloxacino.

Con todos estos resultados obtenidos en el laboratorio, el enrofloxacin en su forma comercial (Baytril® 10%) se presentó como un interesante candidato a estudiar en granja por las potenciales diferencias que puede haber en función del genotipo bovino en la secreción a leche y distribución plasmática. El diseño experimental consistió en la administración de Baytril® 10% a una dosis de 2,5 mg/kg a cinco vacas Y/S y a seis vacas Y/Y. Los parámetros farmacocinéticos plasmáticos obtenidos (Tabla 1, artículo III) fueron similares a los obtenidos para esa misma dosis en los estudios de McKellar et al., (1999). En lo que respecta a las diferencias entre los genotipos, las AUCs plasmáticas fueron significativamente inferiores para las portadoras del genotipo Y/S ($1,86 \pm 0,32 \mu\text{g}\cdot\text{h/ml}$) que para las portadoras del genotipo Y/Y ($2,33 \pm 0,23 \mu\text{g}\cdot\text{h/ml}$) (Tabla1, artículo III). Aunque en los estudios previos se había observado una tendencia, hasta el momento no se había demostrado que el polimorfismo bovino Y581S afectara a los niveles plasmáticos de sus sustratos. Este resultado está en línea con la actividad general del transportador que tradicionalmente se ha visto involucrado en la absorción y secreción sistémica de fármacos. Por lo tanto, la mayor actividad de la variante polimórfica Y581S podría promover una más rápida y eficiente eliminación de algunos de sus sustratos a través de la orina (principal vía de eliminación del enrofloxacin (Martinez et al., 2006)), la bilis, el lumen intestinal y la leche, dando lugar a una reducción en los niveles plasmáticos de las vacas polimórficas. Aunque en el caso del ciprofloxacino los niveles plasmáticos también son menores en las vacas Y/S (Tabla 1, artículo III), no se puede achacar, al menos totalmente, a un efecto directo del polimorfismo, sino posiblemente también a un reflejo de las diferentes concentraciones de partida en plasma del enrofloxacin, el metabolito parental que pasa a ciprofloxacino principalmente en el hígado. De hecho,

las diferencias en las AUCs plasmáticas de ciprofloxacino entre los dos grupos para ambos fármacos es similar a la del enrofloxacin, en torno a un 20%.

En lo que respecta a las diferencias obtenidas en los parámetros farmacocinéticos de la leche en función del genotipo también observamos un efecto producido por las variantes genéticas del transportador. Al igual que en los estudios previos con el danofloxacin, observamos unos valores de AUCs y del ratio AUCs leche/plasma significativamente mayores en los animales Y/S en comparación con los animales Y/Y (Tabla 2, artículo III). Los ratios AUCs leche/plasma fueron 1,5 veces mayores para el enrofloxacin ($0,52 \pm 0,03$ en las vacas Y/Y frente $0,84 \pm 0,15$ en las vacas Y/S) y 2,5 veces mayor para el ciprofloxacino en las vacas Y/S en comparación con las vacas Y/Y ($3,00 \pm 1,19$ en las vacas Y/Y frente $7,79 \pm 0,66$ en las vacas Y/S). Estas diferencias son similares a las obtenidas en los estudios previos con el danofloxacin.

Por otro lado los valores del ratio AUCs leche/plasma del ciprofloxacino son hasta 9 veces mayores que los del enrofloxacin (Tabla 2, artículo III), lo cual indica que el ciprofloxacino es mucho más eficientemente acumulado en leche que el enrofloxacin. Independientemente del genotipo, el perfil terapéutico en leche está dominado por el ciprofloxacino (Figura 4, artículo III). Estas mismas diferencias en las concentraciones en leche de ambos compuestos ya habían sido indicadas previamente por otros autores (Kaartinen et al., 1995; Rantala et al., 2002; Idowu et al., 2010). Se cree que esta diferencia se puede deber a que parte del enrofloxacin se puede metabolizar a ciprofloxacino en la ubre. Por otro lado también se podría deber a que el bajo pH de la leche ionizaría el ciprofloxacino y como consecuencia esta molécula quedaría confinada en la leche (Malbe et al., 1996). No podemos descartar que parte de las diferencias en las concentraciones en leche obtenidas en el ciprofloxacino entre genotipos provengan de las diferencias en las concentraciones de enrofloxacin. Sin embargo, la diferencia de los ratios AUCleche/plasma para el enrofloxacin entre genotipos es 1,5 veces mayor para las vacas Y/S, mientras para el ciprofloxacino es

2,5 mayor para estas vacas, indicando un efecto adicional dependiente del genotipo para el ciprofloxacino y que no podría explicarse exclusivamente por la metabolización de enrofloxacin en ciprofloxacino.

Algunos estudios han sugerido que los polimorfismos pueden afectar a la expresión de determinados transportadores incluso en el caso de individuos heterocigotos (Wojnowski and Brockmoller, 2004; Visser et al., 2014). Para determinar si la diferencia en la secreción de antimicrobianos se debe a una mayor actividad del transportador o a una mayor expresión del mismo según el genotipo se llevó a cabo un estudio de expresión de RNA y proteína mediante qRT-PCR y *western blot*, respectivamente, a partir de glóbulos grasos de leche procedente de dos grupos de animales Y/S y Y/Y. Atendiendo a los resultados obtenidos derivados del estudio de la expresión de RNA de las dos variantes genéticas del ABCG2 bovino la variabilidad obtenida impide la obtención de conclusiones (Figura 4a, artículo II). Sin embargo, los estudios de proteína obtenidos de la membrana de las células alveolares mamarias (Figura 4b, artículo II), mostraron una expresión proteica muy similar en ambos grupos, indicando que el polimorfismo Y581S no induce la expresión del transportador por lo que, al igual que se había concluido en los estudios *in vitro*, nuestros experimentos confirmaron por primera vez que el polimorfismo Y581S es un polimorfismo de ganancia de función *in vivo* que incrementa la capacidad del transportador bovino ABCG2 de transportar sus sustratos.

El conocimiento de los factores involucrados en la concentración de fármacos en leche es esencial para predecir de manera más precisa la contaminación inesperada de productos lácteos o el fracaso de tratamientos, por lo que estos resultados son un nuevo paso en la identificación de todos aquellos factores que tienen importancia en la variabilidad interindividual y la respuesta a un tratamiento farmacológico, lo que permitiría un mejor ajuste de dosis y de los periodos de supresión. Además dado que las fluoroquinolonas estudiadas tienen un efecto dosis dependiente, se puede dar el

caso de que su biodisponibilidad se vea afectada específicamente en algún tejido diana, no llegando a su concentración mínima inhibitoria, pudiendo favorecer así el desarrollo de resistencias (Illambas et al., 2013). Por otra parte, esta diferencia de transporte debida al polimorfismo, no tiene por qué ser exclusiva de antibióticos; también puede hacerse extensivo a otras toxinas presentes en el organismo derivadas de la dieta que ya se han descrito como sustratos del transportador ABCG2. Entre ellas están la toxina fúngica aflatoxina B1, o la fototoxina feoforbida (Wassermann et al., 2013a).

6.1.2. Influencia del polimorfismo en la secreción láctea de compuestos de interés nutricional

Dentro del grupo de moléculas que ya han sido descritas como sustratos del transportador ABCG2 para las variantes murina y humana existen algunas sustancias de origen natural cuya relevancia puede ser clave para la industria láctea como el ácido úrico y la riboflavina (Vitamina B₂).

El ácido úrico secretado a leche posee un potencial antioxidante similar al de los carotenoides, el ácido ascórbico y el α -tocoferol, confiriendo un papel importante en lo que respecta a la calidad de la leche y sus propiedades antioxidantes (Larsen y Moyes, 2010), pudiendo afectar al potencial redox, a pesar de que éste depende también de otras moléculas como el ácido ascórbico y el α -tocoferol (Aycicek et al., 2006).

Asimismo, la dieta que reciben las vacas es otro factor que determina la capacidad nutritiva de la leche. Los lignanos son unos polifenoles que se pueden encontrar en la práctica totalidad de las especies del Reino Vegetal. El lino (*Linum usitatissimum*) es la fuente de lignanos vegetales más abundante con elevadas cantidades del precursor de enterolignanos secoisolariciresinol diglucósido (SDG) (Axelson et al., 1982). La enterolactona es un metabolito derivado del lignano secoisolariciresinol producido por

la microbiota ruminal e intestinal, que se ha encontrado en concentraciones considerables en la leche (Antignac et al., 2004). Cuando las vacas se alimentan con semillas de lino y *Trifolium repens* acumulan una gran cantidad de enterolactona en leche (Steinshamn et al., 2008; Petit et al., 2009). La enterolactona posee actividad antioxidante por lo que la ingesta de lignanos y sus derivados como parte de una dieta sana, parece tener un carácter protector frente a enfermedades cardiovasculares, diabetes, síndrome metabólico, cáncer, estrés oxidativo e inflamación (Adolphe et al., 2010). Además, la ingesta de lignanos y derivados en la dieta se ha descrito como un mecanismo de defensa frente a determinadas enfermedades crónicas (Adolphe et al., 2010).

Por último, van Herwaarden et al., (2007) también ha demostrado el papel de ABCG2 en la secreción a leche de la riboflavina (vitamina B₂) con el evidente interés e impacto nutricional que ello puede tener para la industria de los lácteos.

Por ello, decidimos hacer un estudio detallado del efecto del polimorfismo bovino Y581S de ABCG2 en la secreción a leche de estos compuestos: el ácido úrico, la enterolactona y la riboflavina que por sus propiedades químicas o nutritivas tienen una especial relevancia para la industria láctea (artículo IV). Los modelos generados con las variantes bovinas Y581 y S581 en células MDCK-II han demostrado ser adecuados para la predicción de la secreción de compuestos a leche y pueden ayudar a dirimir qué factores hay detrás de las variaciones interindividuales. Sin embargo, cómo afecta la modificación del contenido de determinados compuestos mediada por ABCG2 a la calidad de la leche aún no se puede predecir con exactitud. Este es el caso del ácido úrico, un metabolito purínico que es capaz de actuar como un potente antioxidante. Nuestros estudios *in vitro* confirmaron que el ácido úrico es sustrato del ABCG2 bovino (Tabla 2, artículo IV). Además, los resultados *in vivo* concluyeron que las vacas Y/S presentaban una concentración de ácido úrico mayor que las vacas Y/Y en leche ($421,1 \pm 54,2 \mu\text{M}$ para las vacas Y/S frente $254,4 \pm 26,3 \mu\text{M}$ para las vacas Y/Y (Figura

3, artículo IV)). Estos estudios apuntan a que ABCG2 transporta úrico a la leche y además que la concentración de ácido úrico es mayor en la leche de las vacas Y/S. Dado que las concentraciones fisiológicas de ácido úrico han demostrado que pueden inhibir al transportador (Dankers et al., 2013), es un factor a tener en cuenta en el estudio de la secreción diferencial de compuestos a leche.

La riboflavina también ha sido testada en nuestro modelo *in vitro* ya que es un micronutriente relevante en una gran cantidad de procesos biológicos. Estudios previos de van Herwaarden et al. (2007) demostraron que esta vitamina es sustrato de la isoforma humana y murina del transportador. Además descubrieron que el transportador limitaba la biodisponibilidad de la riboflavina en plasma y órganos y que además era activamente secretada a leche a través de esta proteína. Sin embargo, nunca antes se ha demostrado cuál es su papel a este respecto en rumiantes. El transporte transepitelial en células polarizadas MDCK-II nos ha permitido identificar a la riboflavina como un sustrato del ABCG2 bovino. Estos resultados también demostraron la presencia de un mecanismo basolateral de transporte de riboflavina, lo cual está en concordancia con estudios previos que apuntan a la existencia de transportadores específicos de riboflavina en la membrana basolateral de las células (Yonezawa and Inui, 2013). Sin embargo, nuestros estudios demuestran la presencia de otro mecanismo de transporte mediado por ABCG2 bovino en la membrana apical. De acuerdo con nuestros resultados *in vitro* con células MDCK-II, la variante S581 es más eficiente transportando vitamina B₂ que la variante *wild-type* ya que revierte en mayor medida el transporte basolateral de riboflavina (Figura 4, artículo IV).

La relevancia de este transportador en la concentración de esta vitamina en leche viene cuestionada por la ausencia de efecto en las crías de ratones hembras *Abcg2*^(-/-) alimentadas con una dieta deficiente en riboflavina, al menos en un entorno de laboratorio. En cualquier caso, el estudio de los factores que pudieran afectar a la composición vitamínica de la leche presenta un gran interés para la industria láctea. El

análisis de la leche procedente las vacas portadoras y no portadoras del polimorfismo no reveló diferencias entre ambos grupos experimentales, probablemente debido a que el efecto en células que sobreexpresan el transportador es más acusado que el de un sistema *in vivo* más complejo y realista como es una explotación ganadera con individuos heterocigotos. Además existen otros mecanismos alternativos o compensatorios que pueden ocultar una diferencia en la acumulación de esta vitamina como la implicación de los cofactores FAD y FMN, precursores de la vitamina (van Herwaarden et al., 2007).

En lo que respecta a la interacción de la enterolactona con el ABCG2 bovino el estudio de niveles de enterolactona en la leche y plasma de las vacas demostró que son comparables con las concentraciones obtenidas en otros trabajos con estos rumiantes (Antignac et al., 2004; Gagnon et al., 2009). La enterolactona se considera otro factor que puede promover un ambiente antioxidante en la glándula mamaria (Cortes et al., 2012; Schogor et al., 2013). Nuestros estudios *in vitro* han demostrado que la enterolactona es un potente y específico sustrato del ABCG2 bovino y además que es un sustrato transportado de manera más activa por la variante S581 del transportador bovino ABCG2 (Tabla I, artículo IV). En rumiantes, los lignanos son metabolizados en el rumen y están disponibles para su absorción en el intestino delgado como enterolignanos (Petit et al., 2009). Los estudios de Njastad et al., (2014) apuntan que la concentración de este compuesto en leche puede estar limitada por procesos de reconjugación y secreción en el rumen la pared intestinal. Recientes estudios localizan a ABCG2 bovino en rumen, intestino e hígado (Lindner et al., 2013; Haslam and Simmons, 2014) por lo que no es de extrañar que ABCG2 también tomara parte en el proceso de secreción a leche y al mismo tiempo de limitación sistémica a través de la conjugación ya que se ha demostrado un proceso similar con ciertas isoflavonas, sustratos naturales del transportador derivados también de la dieta (Alvarez et al., 2010). Los niveles plasmáticos de enterolactona obtenidos en las vacas

Y/S son mucho menores que en las vacas Y/Y (Figura 2, artículo IV), lo cual indica el importante papel del polimorfismo en la disposición plasmática de enterolactona. El ratio leche/plasma es mucho mayor en vacas Y/S que en las Y/Y ($2,99 \pm 0,46$ para las vacas Y/S frente $1,44 \pm 0,50$ en las vacas Y/Y) corroborando el carácter de ganancia de función del polimorfismo de nuestro transportador.

Además de ser sustrato de ABCG2 la enterolactona presenta la capacidad de inhibir al transportador. Los experimentos *in vitro* realizados con enterolactona a 100 y 200 μM fueron capaces de revertir la acumulación de mitoxantrona hasta en un 70% (Figura 1, artículo IV). La variante S581 presentó una menor inhibición del transporte de mitoxantrona probablemente debido a que esta variante tiene una mayor capacidad de transporte y por lo tanto su actividad es más difícilmente inhibible. Este mismo efecto ha sido descrito en estudios previos con otros inhibidores como la selamectina o el triclabendazol sulfóxido y sulfona (Merino et al. 2009; Barrera et al., 2013).

6.2. La enterolactona como sustrato y modulador del transportador ABCG2

6.2.1. Estudios en cultivos celulares y ratones *wild-type* y *Abcg2*^(-/-)

Tras los excelentes resultados obtenidos con la enterolactona y el ABCG2 bovino, nos planteamos el estudio de su interacción con Abcg2 murino como modelo preclínico y con ABCG2 humano (artículo V). El interés por todos estos compuestos ha aumentado en los últimos años, como parte de la investigación relacionada con los efectos derivados de la acción de determinados nutrientes en la prevención y el control del cáncer (Adlercreutz, 2002; Del Rio et al., 2013). En este sentido, la enterolactona y el enterodiol se consideran los enterolignan más activos biológicamente. Debido a estas propiedades beneficiosas sobre la salud, de un tiempo a esta parte, han ido apareciendo en el mercado diversos productos a base de cáscara de semilla de lino, los cuales se emplean fundamentalmente como complementos alimenticios. Dado que

no se había demostrado la interacción directa de la enterolactona como sustrato del transportador ABCG2 humano y murino se realizaron estudios usando células MDCK-II transducidas y ratones *wild-type* y *Abcg2*^(-/-) para dilucidar el papel de ABCG2 en la biodisponibilidad del lignano enterolactona y estudiar de forma directa su acción en su transferencia a la leche.

Los resultados obtenidos en nuestro estudio evidencian una actividad directa de ABCG2 en el transporte de la enterolactona. Los experimentos de transporte transepitelial utilizando células MDCK-II parentales y células MDCK-II transducidas con el transportador murino y humano demostraron que la enterolactona es translocada en sentido apical en las células que sobreexpresan el transportador con un ratio de transporte basal-apical/apical-basal de 16,5 y 3,1 para la variante murina y humana respectivamente. Este transporte fue revertido bajo el efecto del inhibidor específico de ABCG2 Ko143 (Figura 1, artículo V) confirmando *in vitro* el papel específico de este transportador en el transporte de enterolactona. Estudios previos han demostrado que a concentraciones en el rango μ molar, la enterolactona tiene la capacidad de modular diferentes componentes de ciertas cascadas de señalización intracelular de crecimiento, proliferación o apoptosis (Crozier et al., 2009; Corsini et al., 2010). Por lo tanto nuestros resultados pueden tener una importante significación fisiológica en aquellos órganos donde hay una elevada expresión del transportador como el intestino, el sincitiotrofoblasto y la glándula mamaria (Mao and Unadkat, 2015).

Para confirmar la relevancia de estos resultados *in vivo*, se analizaron los niveles plasmáticos y de leche endógenos del enterolignano enterolactona en ratones, demostrando el papel de *Abcg2* en la secreción a leche de este compuesto. El ratio leche/plasma de este compuesto en los ratones *Abcg2*^(-/-) fue mucho menor que el de los ratones *wild-type* ($0,4 \pm 0,1$ frente a $6,4 \pm 2,6$) (Tabla 1, artículo V), lo cual indica que los ratones *Abcg2*^(-/-) al carecer del transportador pierden la capacidad de

concentrar este metabolito en la leche en más de un 90% de lo que lo hacen los ratones *wild-type*. Las concentraciones medias en plasma de los ratones son similares a las obtenidas en humanos (57 ± 13 nM) (Tabla 1, artículo V).

Se ha demostrado previamente que la disposición de las formas conjugadas de lignanos que se forman en el intestino y en el hígado también dependen de los transportadores ABCC2 y ABCG2 (Lampe et al., 2006; Krumpochova et al., 2012; van de Wetering y Sapth, 2012). Estos autores demostraron que la enterolactona sulfato es un sustrato de baja afinidad de ABCG2 y que los ratones *Abcg2*^(-/-) presentan alteraciones en los niveles de enterolactona sulfato, demostrando la relevancia de este transportador en la disposición general no solo de la enterolactona, sino también de sus derivados. Estudios de alimentación controlada en rumiantes han demostrado que la excreción de lignanos en la orina y en la leche es dosis-dependiente (Lampe et al., 2006; Petit et al., 2009), por lo tanto la interrelación de la enterolactona y sus conjugados con ABCG2 así como la microbiota intestinal son factores esenciales que afectan a la disposición global de la enterolactona.

Para completar la caracterización de este compuesto se estudió la capacidad de la enterolactona para modular la actividad del transportador ABCG2 murino y humano. Los estudios de acumulación de mitoxantrona mediante citometría de flujo con células MDCK-II parental y transducidas con los transportadores ABCG2 murino y humano demostraron que la enterolactona era capaz de inhibir el transporte de mitoxantrona incrementando la acumulación intracelular de este compuesto hasta un 38% en presencia de enterolactona 200 μ M (Figura 2, artículo V). Dado que el rango fisiológico de la enterolactona se encuentra entre 10 y 1000 μ molar en el lumen del colon (Kitts et al., 1999) y dado que de acuerdo con nuestros experimentos esas concentraciones son capaces de inhibir la actividad del transportador, una eventual exposición a dietas ricas en lignanos puede tener implicaciones fisiológicas y farmacológicas debido a las interacciones con otros sustratos. Además estos resultados pueden ser de interés en

terapias que pretenden revertir la resistencia en tratamientos quimioterapéuticos (Allen et al., 2002; Robey et al., 2009).

6.2.2. Modulación de la actividad del transportador ABCG2 ovino a través de dietas enriquecidas en lino: impacto sobre la secreción de danofloxacino a leche

Las evidencias de la existencia de interacciones entre fármacos convencionales y ciertos productos naturales derivados de los vegetales están cada vez más extendidas y son cada vez más habituales en la práctica clínica veterinaria. Para evaluar estas interacciones, las técnicas tradicionalmente utilizadas para estudiar las interacciones fármaco-fármaco pueden ser readaptadas para el estudio de este nuevo tipo de interacciones (Brantley et al., 2014) .

En los últimos años han proliferado los estudios en relación a los efectos beneficiosos de la suplementación de semillas de lino en dietas de rumiantes, y cómo estas dietas potencian la presencia de los metabolitos derivados del SDG en fluidos biológicos (Antignac et al., 2004; Cortes et al., 2013; Schogor et al., 2014). Las semillas de lino y sus derivados son un suplemento alimenticio considerado como una fuente aceptable de energía y proteína (Schogor et al., 2014). La enterolactona se produce principalmente en el rumen por *Prevotella spp.* que biotransforma el SDG y promueve así su absorción (Schogor et al., 2014). Tras la ingesta, el SDG es biotransformado en secoisolariciresinol (SECO) y posteriormente la microbiota ruminal e intestinal transforma el SECO en sus metabolitos enterodiol y enterolactona (Petit et al., 2009). Por ello, y tras los excelentes resultados previos en cuanto a la inhibición *in vitro* del transportador ABCG2 bovino (Figura 1, artículo IV) y humano y murino (Figura 2, artículo V) por parte de la enterolactona, en el caso que nos ocupa consideramos necesario estudiar la posibilidad de interacciones fármaco-alimentarias con los derivados del SDG que pueden afectar a la farmacocinética y secreción en leche de determinados compuestos sustratos del transportador en ovejas.

Interacciones de este tipo ya se han descrito con otros compuestos derivados de la dieta como las isoflavonas (Pulido et al., 2006; Vlaming et al., 2009; Perez et al., 2013). Estudios previos con el danofloxacino y el enrofloxacino demostraron que dietas enriquecidas en derivados de la soja eran capaces de actuar sobre la actividad del transportador ABCG2 en ovejas y a través de su inhibición reducir la secreción de estos compuestos a leche en más de un 30% (Pulido et al., 2006; Perez et al., 2013).

Para probar la posibilidad de interacciones fármaco-alimentarias con los derivados de SDG, se realizó un estudio *in vitro* de transporte transepitelial de danofloxacino con enterolactona, el cual demostró que la enterolactona es un eficiente inhibidor del transporte de danofloxacino. A una concentración de 100 μ M de enterolactona el transporte de danofloxacino mediado por el ABCG2 ovino revirtió casi por completo pasando de un ratio de transporte basal-apical/apical-basal de $9,22 \pm 0,69$ a $1,36 \pm 0,11$, que es un ratio similar al obtenido en las células parentales que carecen del transportador (Figura 1, artículo VI).

A continuación, se llevó cabo un estudio farmacocinético de danofloxacino en presencia de dietas enriquecidas con semillas de lino en ovejas. Estas dietas suplementadas con el precursor de lignanos SDG conllevan la acumulación plasmática y en leche de sus derivados metabólicos enterolactona y enterodiol. Existen múltiples estudios que han valorado la cantidad de enterolactona y enterodiol en plasma y leche de vaca tras una suplementación de la dieta con lignanos (Cortes et al., 2013). En cambio, es la primera vez que se hace un estudio de este tipo en ovejas. Al igual que sucede en bóvidos (Gagnon et al., 2009), demostramos que existe una correlación entre la cantidad de lignanos en la dieta y la cantidad de enterolactona en plasma y leche de oveja tras suplementar una dieta durante dos semanas (Figura 2, artículo VI).

Tras la suplementación de las dietas con 15%, 10% y sin lino durante 15 días, se administró a los animales danofloxacino 1,25 mg/kg intramuscular. Los perfiles plasmáticos obtenidos fueron similares a los obtenidos por Aliabadi et al. (2003) y

Barrera et al. (2013) y no se observó ninguna diferencia entre los tres grupos de animales. Con respecto a los resultados de secreción de danofloxacino a leche, los resultados del grupo control fueron muy similares a los obtenidos por Barrera et al. (2013) y Perez et al. (2013). En las ovejas que habían recibido lignanos en la dieta la secreción de danofloxacino se redujo entre un 25-30% (Figura 4, tabla 3, artículo VI), lo cual parece indicar que en la glándula mamaria se da un fenómeno de competencia entre el danofloxacino y la enterolactona por el transportador ABCG2 de manera muy similar al observado *in vitro*. Los resultados no mostraron diferencias entre las dietas enriquecidas al 10 y 15 % en lignanos, probablemente debido a que las elevadas concentraciones obtenidas en plasma producen una saturación del transporte de enterolactona a leche mediado por ABCG2. Las concentraciones similares de danofloxacino obtenidas en leche de ambos grupos a pesar de la diferente concentración de enterolactona en plasma de ambos grupos refuerza esta hipótesis de saturación del transporte de enterolactona a leche (Figura 2, artículo VI).

No podemos descartar que otros derivados de los lignanos como el enterodiol (también detectado en plasma y leche de oveja (Figura 2, artículo VI)), y los conjugados del enterodiol y de la enterolactona puedan tener un efecto adicional en lo que respecta a la modulación del transporte de danofloxacino mediado por ABCG2. De hecho, se ha descrito la conjugación de la enterolactona a nivel hepático con glucurónidos y sulfatos (Gagnon et al., 2009). En cualquier caso, la enterolactona es el lignano más abundante en leche de vaca tras el enriquecimiento en lino de las dietas (Antignac et al., 2004) por lo que lo más probable es que cualquier efecto derivado de la acción de lignanos sobre el transportador ABCG2 esté dominado por la enterolactona aun descartando la hipótesis que sea el único modulador proveniente de la dieta. No obstante, para comprender el alcance del efecto sobre ABCG2 de todos los compuestos derivados de la ingesta del lino son necesarios estudios adicionales.

Las dietas enriquecidas en lino reportan una serie de propiedades nutritivas y antioxidantes a la leche muy interesantes a través de enterolactona, su metabolito principal. Además del valor añadido que supone para el producto por sus beneficios, su capacidad para inhibir el transportador ABCG2 se presenta como una alternativa más adecuada a la inhibición por medio de otros xenobióticos que habitualmente producen una serie de efectos secundarios indeseables (Mao and Unadkat, 2015).

7. ANEXO I

Estudio de las propiedades fisicoquímicas de la leche y análisis metabolómico comparativo del plasma, leche y orina de vacas portadoras y no portadoras del polimorfismo Y581S del transportador ABCG2.

7.1. Introducción

En las secciones anteriores hemos demostrado un efecto drástico del polimorfismo bovino Y581S de ABCG2 sobre la distribución de agentes xenobióticos pero también sobre compuestos endógenos o procedentes de la dieta como el ácido úrico y la enterolactona. Estos compuestos pueden tener actividades antioxidantes y afectar a las propiedades fisicoquímicas de la leche y a su procesamiento industrial posterior por lo que un análisis de dichos parámetros puede aportar información importante sobre el efecto del polimorfismo sobre la calidad de la leche. Así pues, para completar la caracterización de este polimorfismo *in vivo* se realizó un estudio de las propiedades fisicoquímicas de la leche así como un estudio metabolómico de la leche producida por vacas portadoras y no portadoras del SNP Y581S, para analizar si existen diferencias en las propiedades de la leche producida por ambos grupos.

7.2. Análisis de las propiedades fisicoquímicas de la leche procedente de vacas Y/Y y vacas Y/S

7.2.1. Materiales y métodos

Grupos experimentales

El manejo y alojamiento de los animales se llevó a cabo de acuerdo con la legislación europea vigente (2010/63/EU). Para realizar los ensayos se utilizaron vacas Holstein de entre 2 y 5 años de edad y de entre 600 y 800 kg de peso. Las vacas utilizadas eran ordeñadas dos veces al día y la producción diaria media de leche era de 41 ± 7 kg. Los animales estaban libres de parásitos y disponían de comida y bebida *ad libitum*. La recogida de muestras se llevó a cabo en la granja Garfi SAT de Santa María del Monte del Condado, León (España). El genotipado de los animales se llevó a cabo según lo descrito en Otero et al. (2015). Los animales se dividieron en dos grupos de 6 vacas Y/S y 7 vacas Y/Y. La toma de muestras se realizó en el ordeño matutino tras descartar el primer minuto de ordeño y realizar el ordeño completo de la

ubre. Las muestras fueron inmediatamente trasladadas a la Estación Tecnológica de la leche de Palencia (Instituto Tecnológico Agrario, Junta de Castilla y León, España) en condiciones de refrigeración.

Análisis de las propiedades fisicoquímicas de la leche

Estos análisis se llevaron a cabo por el grupo del Dr. Luis Rodríguez Ruiz en la Estación Tecnológica de la leche de Palencia (Instituto Tecnológico Agrario, Junta de Castilla y León, España).

Determinación del color. Se determinaron y evaluaron las coordenadas de color en el espacio CIELAB: L (luminosidad) y a^* , b^* (cromaticidad), utilizando un colorímetro CR-410 (Konica Minolta, España). Las condiciones de operación fueron: observador 10° , Iluminante D65. Para diferenciar las coordenadas cartesianas rectangulares de a^* y b^* se emplean las coordenadas cilíndricas para obtener la saturación (Factor C^*) y la tonalidad (Factor H). El parámetro de luminosidad (L^*), definido como el atributo según el cual una superficie parece emitir más o menos luz y que varía entre 0 (negro absoluto) y 100 (blanco absoluto) (Gilbert, E. (1992). Medida del color. Valencia: Servicio de publicaciones UPV). El parámetro a^* es la desviación del punto cromático, en el que si a^* es mayor a 0 tiende hacia el color rojo y hacia el verde si es menor a 0 (Gilbert, 1992). El parámetro b^* corresponde también a una coordenada de cromaticidad, pero en este caso va hacia amarillo si es mayor a 0 y hacia azul si es menor a 0 (Gilbert, 1992). El Croma C^* es igual al módulo del vector que une el punto representativo del color, proyectado sobre el plano a^* , b^* con el centro de coordenadas, es decir, la distancia entre el centro y el punto proyectado sobre el plano de cromaticidad. El ángulo de tono H^* es aquel que forma el vector del punto que representa el color proyectado sobre el plano de cromaticidad a^* , b^* con el eje a^* positivo.

Determinación de la viscosidad. El comportamiento reológico de la leche se estudió con un reómetro PHYSICA MCR 301 (Anton Paar GMBH, Austria), mediante sistema de cilindros coaxiales CC27 acoplado a un sistema peltier para cilindros C-PTD200. Los datos se controlaron con el programa Rheoplus (Anton Para GMBH, Austria), instalado en el ordenador conectado al reómetro.

Determinación del índice de refracción. Se llevó a cabo mediante un refractómetro modelo: Digital Hand-held "Pocket" Refractometer PAL-RI/PAL-RI (SUS). ATAGO.

Determinación de cationes en leche. Se determinaron sodio, potasio, calcio y magnesio mediante cromatógrafo iónico 882 Compact IC, columna Metrosep C4 150 y software MagIC 1.1 (Metrohm, Herisau, Suiza).

Determinación del pH. Se llevó a cabo mediante un pH METER GLP 22. CRISON. Se trata de un pH-metro con display digital con resolución de 0,01 unidades de pH y compensación de temperatura manual o automática. Electrodo combinado de vidrio, apto para un rango de al menos 3 a 12 unidades de pH.

Determinación de la conductividad. Se llevó a cabo mediante el conductivímetro modelo HI 9033N MultiRange ConductivityMeter Hanna instruments, con una sonda HI 76302W calibrada para un rango de 0,00 a 19,99 mS/cm.

Determinación del potencial redox. Se determinó mediante un potenciómetro modelo 744 pH Meter Metrohm. Se trata de un electrodo de anillo de Pt combinado LL, EME-12-ACC-02 Metrohm 00962254.

Determinación de la densidad. Se determinó mediante un Lactodensímetro Quevenne lectura en haut Tp. 20°C. Gerber Instruments AG 05.1020-20°C. 8307 Effretikon Switzerland.

Determinación de la acidez (método Dornic). Se determinó mediante titración con una bureta digital Titrette 25ml class A Brand (Wertheim, Alemania) hasta pH 8.30

determinado con pHmetro GLP 22 y electrodo combinado con compensación automática de temperatura 5053 Crison (Barcelona, España).

Determinación de sustancias inhibidoras. Se determinó mediante procedimiento de trabajo: PE/LILCYL/11 “Procedimiento para la detección de sustancias inhibidoras (Método Eclipse)”. Acreditado por ENAC. Es un método basado en inhibición bacteriana. (Z.E.U- INMUNOTEC®) Se utilizan métodos analíticos de cribado para la determinación de sustancias inhibidoras. Estos métodos están basados en el cultivo de un microorganismo en agar que tiene resistencia o sensibilidad frente a un antibiótico o grupo de antibióticos determinados.

Recuento de gérmenes totales. Se determinó mediante citometría de flujo, con el Bactoscan FC, fabricado por la empresa Foss Electric o con el Bactocount IBC, fabricado por la empresa Bentley Instruments, procedimiento de trabajo: PE/LILCYL/09 “Procedimiento para el recuento de gérmenes totales”. Acreditado por ENAC.

Determinación de Grasa, proteína, lactosa, sólidos totales, caseína, urea y crioscopía. Se determinó mediante Espectroscopía IR transformada de Fourier (FTIR), con el Milkoscan FT+, fabricado por la empresa Foss Electric, mediante el procedimiento de trabajo: PE/LILCYL/07 “Procedimiento para la determinación de grasa, proteína, lactosa, extracto y punto crioscópico por I.R.”. Acreditado por ENAC. Equipo de infrarrojos (Milko-Scan).

Determinación de Células Somáticas. Se determinó mediante citometría de flujo, con el Fossomatic FC, fabricado por la empresa Foss Electric, mediante el procedimiento de trabajo: PE/LILCYL/08. “Procedimiento para el recuento de células somáticas. Método Fossomatic”. Acreditado por ENAC.

7.2.2. Resultados y discusión

El estudio de las propiedades fisicoquímicas de la leche mostró claras diferencias en la conductividad y la concentración de lactosa de ambos grupos (Tabla 1), con una conductividad más alta en las vacas Y/S y una mayor concentración de lactosa en las vacas Y/Y. Se observó una tendencia que parecía indicar un mayor potencial redox en la leche de las vacas Y/S aunque no se alcanzaron diferencias significativas ($p=0,1$).

De los múltiples parámetros estudiados al analizar las propiedades fisicoquímicas de la leche, los análisis estadísticos revelaron que la conductividad era en torno a un 20% superior en las vacas portadoras del polimorfismo Y/S en comparación con las vacas Y/Y (Tabla 1). Niveles excesivamente elevados de conductividad se relacionan con estadios premastíticos como consecuencia del aumento de la presencia de iones intracelulares en leche (Díaz et al., 2012; Gargouri et al., 2014). Por otro lado, se ha publicado que la conductividad es un parámetro sensible a múltiples variables cuyo incremento en leche puede ser provocado por la reducción del contenido de agentes oxidantes en la leche como el peróxido de hidrógeno (Sakai et al., 2010). Como hemos visto en apartados anteriores, las vacas portadoras del polimorfismo son capaces de secretar ciertos antioxidantes a leche de manera más eficiente que las vacas Y/Y, lo que podría reducir la concentración de algunos agentes superóxido como el peróxido de hidrógeno explicando así la más elevada conductividad observada en estas vacas.

El potencial redox es un parámetro con una gran relevancia por su impacto en las propiedades organolépticas y microbiológicas de productos lácteos (Tachon et al., 2010). De hecho y aunque en este estudio no se obtuvieron diferencias significativas en el potencial redox, se observó una tendencia ($p=0,10$), siendo superior en las vacas polimórficas (Tabla 1, anexo I) lo cual también puede ser debido a la presencia diferencial de ciertos antioxidantes como el ácido úrico (Artículo III).

ANEXO I

Tabla 1. Análisis de los parámetros fisicoquímicos de la leche de vacas Y/Y homocigotas e Y/S heterocigotas para el polimorfismo Y581S del transportador ABCG2. Se realizó una prueba T de Student entre los dos grupos. Se observaron diferencias significativas en el contenido de lactosa y la conductividad. *p<0,05.

		Temperatura (°C)	pH	Conductividad (s/cm)	Potencial redox (v)	Densidad (g/ml)	Acidez (°Dormic)	Índice de refracción	Viscosidad (cP)	Color (S.CIELAB)				
										Factor L* (Luminosidad)	Factor a* (+rojo/-verde)	Factor b* (+amarillo/-azul)	Factor H (Tonalidad)	Factor C (Saturación)
Y/S	Media	19,9	6,7	5,7*	81,2	31,7	14,5	1,3	2,5	89,2	-2,2	7,2	-1,3	7,6
	S.D.	0,3	0,1	0,3	3,4	1,1	1,6	0,0	0,3	0,5	0,4	1,6	0,1	1,5
	max	20,4	6,8	6,2	87,5	33,5	16,8	1,3	3,0	89,7	-1,6	10,4	-1,2	10,6
	min	19,5	6,7	5,2	77,5	30,3	12,7	1,3	2,2	88,6	-2,9	6,3	-1,4	6,7
Y/Y	Media	20,1	6,8	4,9*	76,9	32,1	14,5	1,3	2,7	89,7	-2,1	8,1	-1,3	8,4
	S.D.	0,6	0,0	0,2	3,5	1,7	1,0	0,0	0,3	0,6	0,4	1,6	0,1	1,5
	max	21,0	6,8	5,3	82,5	33,8	16,2	1,4	3,2	90,5	-1,7	10,8	-1,2	11,0
	min	19,4	6,7	4,6	73,0	29,0	13,3	1,3	2,2	88,9	-2,8	6,6	-1,4	7,2

		Grasa (%)	Proteína (%)	Extracto seco (%)	Extracto quesero (%)	Flora total (ufc/ml)	Células somáticas (ufc/ml)	Punto crioscópico (°C)	Lactosa (%)	Urea (%)	mg/kg Na	mg/kg K	mg/kg Ca	mg/kg Mg
Y/S	Media	3,7	3,5	8,8	7,2	23,8	196,2	-0,5	4,5*	318,1	482,6	1580,6	1296,0	115,8
	S.D.	1,2	0,5	0,4	1,6	13,3	235,0	0,0	0,2	26,5	116,6	155,3	169,0	17,9
	max	5,5	4,3	9,2	9,8	46,5	661,0	-0,5	4,7	344,5	642,2	1826,9	1542,1	138,1
	min	2,2	2,9	8,3	5,6	11,0	12,0	-0,5	4,2	281,5	303,1	1351,2	1073,3	93,7
Y/Y	Media	4,6	3,5	9,1	8,0	20,8	903,7	-0,5	4,8*	306,3	363,0	1501,7	1375,4	117,9
	S.D.	0,8	0,4	0,3	1,2	15,7	965,9	0,0	0,2	25,6	119,2	127,4	185,1	22,4
	max	6,1	4,0	9,6	10,2	54,0	2647,0	-0,5	5,1	351,0	582,9	1757,2	1601,3	157,7
	min	3,6	2,7	8,6	6,3	5,0	101,5	-0,5	4,4	277,0	271,8	1405,7	1041,9	95,3

El contenido en lactosa fue significativamente superior en las vacas Y/Y comparado con la leche producida por las vacas Y/S (Tabla 1, anexo I). Se sabe que transportadores ABC bacterianos son capaces de transportar disacáridos como la maltosa (Bordignon et al., 2010). En mamíferos sin embargo no se tienen evidencias de que los transportadores ABC puedan transportar carbohidratos como la lactosa por lo que de momento desconocemos cómo el polimorfismo Y581S puede afectar a los niveles de lactosa en leche ya que la producción de esta azúcar es un mecanismo complejo en el que intervienen gran cantidad de factores.

El estudio comparativo del contenido en proteína y grasa entre los dos grupos no reveló diferencia entre ellos, lo cual está en desacuerdo con lo reportado por Cohen-Zinder et al., (2005). Dado que ambos factores están estrechamente relacionados con la dieta, la discrepancia de resultados se puede deber a un efecto de la diferencia del forraje suministrado a los animales de ambos estudios y a ciertas características del manejo animal como el número de ordeños diarios, que afectan drásticamente a las propiedades lácteas. Asimismo, el tamaño muestral ha podido limitar la potencia de nuestro estudio de las propiedades fisicoquímicas de la leche.

7.3. Análisis metabolómico del plasma, leche y orina de vacas Y/Y y vacas Y/S

7.3.1. Materiales y métodos

Grupos experimentales

El manejo y alojamiento de los animales se llevó a cabo de acuerdo con la legislación europea vigente (2010/63/EU). Para realizar los ensayos se utilizaron vacas Holstein de entre 2 y 5 años de edad y de entre 600 y 800 kg de peso. La producción diaria media de leche de las vacas utilizadas era de 41 ± 7 kg y fueron ordeñadas dos veces al día. Los animales estaban libres de parásitos y disponían de comida y bebida *ad libitum*. La recogida de muestras se llevó a cabo en la granja Garfi SAT de Santa

María del Monte del Condado, León (España). El genotipado de los animales se llevó a cabo según lo descrito en Otero et al., (2015). Los animales se dividieron en dos grupos de 5 vacas Y/S y 6 vacas Y/Y. La toma de muestras se realizó en el ordeño matutino. Las muestras de leche se tomaron manualmente de los cuartos delanteros. Las muestras de orina se recogieron estimulando la micción bovina tras el ordeño. Las muestras de sangre se centrifugaron 1000 x g durante 15 minutos y las muestras de plasma, leche y orina se almacenaron a -20°C hasta el análisis.

Análisis preliminar de componentes principales (PCA) del metaboloma de vacas Y/Y e Y/S en plasma y leche a través de LC/MS

100 µl de plasma o leche fueron mezclados con tampón acetato y posteriormente se añadió metanol acidificado con ácido clorhídrico siguiendo el método descrito por Bolca et al., (2010). Las muestras fueron filtradas (0,22 µm) antes de ser inyectadas en el espectrómetro de masas.

Los análisis cuantitativos y cualitativos fueron desarrollados en un “Agilent 1290 Infinity LC system” acoplado a un “6550 Accurate- Mass Quadrupole time-of-flight (QTOF)” (Agilent Technologies, Waldbronn, Alemania).

Los *hits* con una intensidad superior a 50.000 cuentas y detectados entre tiempos de retención específicos fueron considerados para este estudio estadístico y de análisis de componentes principales con el programa informático “Mass Profiler Professional” para determinar diferencias potenciales en el metaboloma de ambos grupos. Este estudio preliminar fue llevado a cabo por el Dr. Juan Carlos Espín y colaboradores en el instituto CEBAS, Murcia (España).

Extracción del metaboloma, análisis y fragmentación de metabolitos a través de LC/MS

Tras descongelar las muestras en hielo, 100 μ L de plasma, leche u orina se desproteinizaron añadiendo 300 μ L de metanol a -20°C . Tras centrifugar ($20.800 \times g$ a 4°C , 10 min), el sobrenadante se evaporó bajo un flujo de nitrógeno gaseoso. Los extractos secos se reconstituyeron en 50 μ L de fase móvil A y se centrifugaron ($20.800 \times g$ a 4°C , 10 min). El sobrenadante se transfirió a viales de HPLC. El análisis cromatográfico se desarrolló con un sistema “Dionex UltiMate 3000 RSLCnano (Thermo Fisher Scientific)”. Se inyectaron 5 μ L de muestra en una columna de HPLC “ReprosilPur Basic C18” ($150 \times 0,5$ mm; $2,4\text{-}\mu\text{m}$) a una temperatura de 30°C . El gradiente utilizado consistió en (0–22 min: 0–53% B; 22–25 min: 53–100% B; 25–35 min: 100% B; 35– 50 min: 0% B) de 5 mM N,N-dimetihexamina (Sigma-Aldrich) en agua:metanol 95:5 (vol:vol) (fase móvil A) y 20:80 5 (vol:vol) (fase móvil B) ajustado a pH 7,0 con ácido acético, a un flujo de 15 μ L/min. La detección se llevó a cabo con un “LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific)” operando en ionización negativa y registrando m/z entre 150 y 1.000 con una resolución de 30.000 FWHM. La ionización por electrospray se realizó a una temperatura de capilar de 300°C . El flujo de gas de cubierta, auxiliar y barrido se ajustó a 20, 10 y 1 unidades arbitrarias respectivamente.

La adquisición de datos se llevó a cabo utilizando el software “Xcalibur (Thermo Fisher Scientific)”. Para detectar diferencias en las muestras de los individuos portadores y no portadores del polimorfismo Y581S, todos los cromatogramas se convirtieron al formato mzXML usando el software Proteowizard y fueron analizados usando las bases de datos XCMS *Online*, HMDB y METLIN. La identidad de los compuestos seleccionados se confirmó comparando los espectros de masas MS/MS con estándares analíticos (Sigma-Aldrich). Para determinar la concentración relativa de los metabolitos seleccionados se generaron cromatogramas y se integraron

manualmente (Jansen et al., 2013). Los análisis de LC/MS/MS fueron realizados por el Dr. Koen van de Wetering y el Dr. Robert Jansen en el *Netherlands Cancer Institute* (NKI), Ámsterdam, Países Bajos.

7.3.2. Resultados y discusión

Análisis preliminar de componentes principales (PCA) del metaboloma de vacas Y/Y e Y/S en plasma y leche

Para determinar la diferencia potencial del metaboloma de vacas Y/Y e Y/S se llevó a cabo un análisis de componentes principales en plasma y leche. Los resultados mostraron una clara agrupación de metabolitos de una manera genotipo-dependiente, sugiriendo una diferencia en la acumulación específica de compuestos a nivel plasmático y en la leche (Figura 7). Tras los análisis estadísticos se encontraron 65 compuestos significativamente diferentes en la leche de ambos grupos y 30 en el plasma.

A) Plasma

B) Leche

Figura 7. PCA de los metabolitos en plasma (A) y leche (B). Los componentes dentro de los círculos rojos corresponden a las vacas Y/Y, mientras que los componentes en los círculos azules corresponden a las vacas Y/S. Cada *score* representa los resultados para un animal concreto (YY o Y/S). Los *loadings* son la aportación de cada compuesto.

Análisis metabolómico del plasma, leche y orina de vacas Y/Y e Y/S

Para concretar el alcance de la relevancia de las diferencias observadas en el estudio de componentes principales se llevó a cabo un estudio detallado del metaboloma en plasma, en leche y en orina con el objeto de determinar algunos de los compuestos diferencialmente concentrados en las vacas portadoras y no portadoras del polimorfismo Y581S.

A) PLASMA

De todos los metabolitos detectados en el plasma de las vacas, solo se representaron aquellos con diferencias significativas entre los dos grupos ($p \leq 0,05$), y con una diferencia en las concentraciones medias igual o mayor del doble (Figura 8). Los análisis del plasma revelaron la presencia de 14 compuestos más concentrados en el plasma de vacas Y/Y no portadoras del polimorfismo. Se detectaron 2 metabolitos significativamente más concentrados en el plasma de vacas Y/S.

Figura 8. Representación en nube de puntos de los metabolitos significativamente distintos detectados en plasma. Los puntos rojos indican los metabolitos con una mayor concentración en las vacas Y/Y y los puntos verdes indican los metabolitos más concentrados en las vacas Y/S.

B) LECHE

De todos los metabolitos detectados en la leche de las vacas, solo se representaron aquellos con diferencias significativas entre los dos grupos ($p \leq 0,05$), y con una diferencia en las concentraciones medias igual o mayor del doble (Figura 9). Los análisis de la leche revelaron la presencia de 47 compuestos más concentrados en la leche de vacas Y/S portadoras del polimorfismo de ABCG2. Se detectaron 5 metabolitos significativamente más concentrados en la leche de vacas Y/Y.

Figura 9. Representación en nube de puntos de los metabolitos significativamente distintos detectados en leche. Los puntos rojos indican los metabolitos con una mayor concentración en las vacas Y/Y y los puntos verdes indican los metabolitos más concentrados en las vacas Y/S.

C) ORINA

De todos los metabolitos detectados en la orina de las vacas, solo se representaron aquellos con diferencias significativas entre los dos grupos ($p \leq 0,05$), y con una diferencia en las concentraciones medias igual o mayor del doble (Figura 10). Los análisis de orina revelaron la presencia de 46 compuestos más concentrados en la orina de vacas Y/S portadoras del polimorfismo de ABCG2. Solamente se detectaron 3 metabolitos significativamente más concentrados en la orina de vacas Y/Y.

Figura 10. Representación en nube de puntos de los metabolitos significativamente distintos detectados en orina. Los puntos rojos indican los metabolitos con una mayor concentración en las vacas Y/Y y los puntos verdes indican los metabolitos más concentrados en las vacas Y/S.

Selección, fragmentación e identificación de metabolitos por espectrometría de masas

Debido al interés nutricional e industrial que posee la leche y la potencial implicación del polimorfismo Y581S en la concentración en leche de algunos compuestos, se seleccionaron algunos de los metabolitos con diferencias significativas en la leche de ambos grupos para su posterior identificación por espectrometría de masas.

Los compuestos a identificar se seleccionaron en base a la intensidad de los picos, al incremento y al grado de significación estadística. Finalmente, 10 compuestos fueron seleccionados para la fragmentación MS/MS. Estos compuestos fueron aquellos con un valor de m/z : 360,9934, 178,0510, 230,0125, 340,1524, 343,1156, 239,9969, 287,0225, 242,0126, 204,0663 y 231,0329. Tras la fragmentación de los metabolitos, los espectros de masas de los metabolitos 178,0510 y 204,0663 coincidieron con el de las bases de datos haciendo posible su identificación. Cabe destacar que el metabolito con m/z 167,021 también pudo ser identificado como ácido úrico a través de las bases de datos, corroborando las diferencias en la concentración de leche que se habían observado en el artículo IV.

El metabolito con m/z 178,0510 se identificó como ácido hipúrico. Su identidad fue confirmada comparando su espectro con el de un estándar analítico.

Figura 11. Espectro de masas del metabolito con m/z 178,0510 detectado en la leche de vacas Y/S a una mayor concentración que en las vacas Y/Y.

El espectro de masas del metabolito con m/z 204,0663 coincidió con el de la N-cinamoilglicina.

Figura 12. Espectro de masas del metabolito con m/z 204,0663 detectado en la leche de vacas Y/S a una mayor concentración que en las vacas Y/Y.

Aunque para los metabolitos con m/z de 230,0125, 239,9969, 287,0225, 242,0126, 231,0329 la identificación no fue posible, todos ellos presentaron tras la fragmentación una pérdida de 79,956 unidades de masa neutra indicando que estos metabolitos estaban conjugados con un grupo sulfato lo que está de acuerdo con la literatura existente que considera a ABCG2 un transportador común de metabolitos sulfatados *in vivo* (van de Wetering and Sapth, 2012).

De igual manera la identificación del metabolito m/z 360,9934 no fue posible. Sin embargo, el espectro de masas muestra la liberación de un fragmento de m/z 167,0211 lo cual indica que es un compuesto relacionado con el ácido úrico, siendo este un estudiadísimo sustrato de ABCG2 (Woodward et al., 2009).

Los estudios metabolómicos realizados por los grupos de investigación del CEBAS (Murcia, España) y del NKI (Ámsterdam, Países Bajos) obtuvieron conclusiones similares, observándose una mayor acumulación de ciertos metabolitos en la leche de las vacas Y/S con respecto a las vacas Y/Y. Asimismo, también se observó que ciertos metabolitos estaban en menor concentración en el plasma de las vacas Y/S. Este efecto apunta en la misma dirección al demostrado tras la administración de

fluoroquinolonas, en los que debido a la mayor actividad de la variante Y581S del transportador hay una mayor secreción a leche de moléculas que al mismo tiempo reduce la cantidad de las mismas en el plasma al ser probablemente eliminadas más eficientemente por los individuos portadores. Este efecto se confirma con el estudio de la orina, a través del cual se observa que del total de metabolitos con concentraciones significativamente diferentes entre las dos variantes, el 90% están más concentrados en la orina de las vacas Y/S.

Dado el especial interés de los componentes de la leche para la industria láctea, de todos los *hits* obtenidos con una mayor concentración en leche de vacas Y/S se pudieron identificar dos de ellos por fragmentación gracias a la espectrometría de masas. Uno de estos candidatos a sustrato con diferencias para portadores y no portadores del polimorfismo Y581S bovino de ABCG2 fue el ácido hipúrico, que se produce como consecuencia de la conjugación de un ácido benzoico con una glicina. La identificación de esta molécula como un potencial sustrato de la variante bovina de ABCG2 viene reforzada por estudios previos que han demostrado que este compuesto tiene la capacidad de inhibir a concentraciones fisiológicas en un 20% el transporte de estrona sulfato mediado por ABCG2 (Mutsaers et al., 2011). Dado que además este compuesto se encuentra en vegetales y frutas (Mutsaers et al., 2011), dietas que favorezcan la formación de ácido hipúrico podrían ser utilizadas para controlar la secreción de otros sustratos de ABCG2 a leche. Hasta el momento no se ha probado que ABCG2 transporte de manera directa esta sustancia aunque presenta un potencial interesante en rumiantes debido a que un incremento en la excreción urinaria de ácido hipúrico es capaz de inhibir las emisiones de N₂O del ganado vacuno ya que retira del organismo el nitrógeno potencialmente metabolizable por las bacterias alojadas en el tracto digestivo de los rumiantes. Dado el problema medioambiental que supone la explotación intensiva ganadera por las emisiones de gas que contribuyen al efecto invernadero, es interesante conocer los factores que pueden afectar las emisiones de

este gas nocivo. Sin embargo, aún es necesario profundizar en cómo el ácido hipúrico reduce las emisiones de N_2O dado que aún no se conoce con detalle cuál es mecanismo que produce este efecto ni cuál es su alcance (Dijkstra et al., 2013). Por otra parte, en nuestro análisis metabolómico de la orina no se pudieron determinar las concentraciones de ácido hipúrico en orina por lo que es necesario llevar a cabo un estudio específico de este compuesto para poder definir mejor las consecuencias biológicas de este hallazgo.

La otra molécula que se pudo identificar es la N-cinamoilglicina. Al igual que al ácido hipúrico este compuesto es un intermediario conjugado con glicina para la eliminación de ácido fenilpropanoico (Rouge et al., 2013). La interacción de este compuesto con ABCG2 podría constituir un sistema del organismo para combatir la toxicidad de compuestos aromáticos a través de la eliminación de los mismos en forma de N-cinamoilglicina. Durante la identificación preliminar de compuestos se detectó que la identidad potencial de multitud de los *hits* analizados eran compuestos conjugados con glicina. Dado que el transportador tiene una alta afinidad por metabolitos en fase II conjugados con sulfatos (van de Wetering and Sapth, 2012) y glucurónidos (Alvarez et al., 2011), este mecanismo podría hacerse extensivo a aquellas moléculas que son conjugadas con glicina como mecanismo celular para su reconocimiento y eliminación. No obstante, para confirmar la relación directa de estas dos moléculas con ABCG2 es necesario realizar en un futuro estudios *in vitro* con nuestro modelo de células MDCK-II que corroboren la existencia de un transporte directo mediado por ABCG2 y con diferencias en el ratio de transporte para ambas moléculas en función del polimorfismo Y581S.

8. SUMMARY

8.1. ABCG2 transporter in the mammary gland and its polymorphisms

The ATP-binding cassette (ABC) transporter ABCG2 (Breast Cancer Resistance Protein) is expressed in a wide range of tissues and organs, including the intestine, liver, blood-brain barrier, and mammary gland (van Herwaarden and Schinkel, 2006) (Figure 3, page 16). It affects the bioavailability of its substrates and mediates the active secretion of xenobiotics and several nutrients into milk (van Herwaarden et al., 2007). The potential occurrence of drug residues in milk could lead to the development of bacterial resistance, allergies, or hypersensitivity reactions in dairy consumers (McManaman and Neville, 2003). Conversely, effective treatments for mastitis may require a considerable transfer of drugs into milk (Escudero et al., 2007). The accumulation of specific nutrients in milk is another factor that can contribute to an improvement of the quality of the products generated in farms. All of these issues are of great concern for general public health, dairy industry, and veterinary therapeutics. Hence, identification of relevant factors for the transfer of drugs into milk in livestock constitutes a priority. Furthermore, changes in the expression and/or the function of ABCG2 can lead to dramatic variations in the pharmacokinetics and secretion of its substrates into milk (Jonker et al., 2007; Ni et al., 2010). In the veterinary field, the discovery of a nucleotide missense adenosine/cytosine mutation encoding a replacement of tyrosine 581 with serine (Y581S) localized on the fifth extracellular region of the bovine ABCG2 transporter constitutes a crucial finding (Wassermann et al., 2013a). Y581S SNP is widely spread in some bovine breeds, reaching a frequency of 20% in the Israeli Holstein population (Ron et al., 2006), and it has been suggested as the causative polymorphism of a quantitative trait locus affecting the production of milk and its protein and fat composition (Cohen-Zinder et al., 2005; Weikard et al., 2012). Therefore, we aimed to investigate whether this polymorphism could affect the capacity of the bovine ABCG2 to transport substances *in vivo*, and if the differences in the transport of specific endogenous substances could be responsible for the differences in milk production described for Y581S SNP carriers. Until now, there are

no *in vivo* studies that corroborate the implications of the *in vitro* results obtained in the laboratory that report differences in the transport of antimicrobials by cells overexpressing both bovine variants (Real et al., 2011b). In this Thesis we will study the *in vivo* the relevance of the bovine Y581S polymorphism of ABCG2 in the bioavailability and secretion of drugs used in veterinary medicine and the effect of this polymorphism in the metabolomic, physicochemical and nutritional properties of milk. Moreover, in order to improve the nutritional properties of milk and to reduce the eventual secretion of drugs into milk, the modulatory effect of specific diets on the activity of ruminant ABCG2 will be evaluated.

8.1.1. Effect of the Y581S polymorphism of the bovine ABCG2 on drug transfer into milk

Our previous *in vitro* results described the Y581S SNP as a gain-of-function polymorphism, showing a lower mitoxantrone accumulation in ovine primary fibroblast transiently transfected with the S581 variant, when compared with the Y581 variant (Merino et al., 2009). Recently, the greater activity of the S581 allele was confirmed on transepithelial transport of antibiotics using stably transduced cell models (Real et al., 2011b). However, there are no studies in ruminants supporting the results obtained in the laboratory. Accordingly, in this Thesis the effect of the polymorphism on the plasma disposition and on milk secretion of multiple drugs widely used in farm has been studied in detail.

Danofloxacin is a fluoroquinolone antibiotic widely used to treat respiratory, digestive tract and mammary gland infections (Schricks and Fink-Gremmels, 2007; Poutrel et al., 2008). Residues derived from treatment with fluoroquinolones are of special interest because they are resistant to thermal treatments, and may persist in dairy products after industrial processing for consumption (Roca et al., 2010). Previous studies by Real et al. (2011a) pointed to danofloxacin as an excellent substrate of ABCG2 transporter with different transport efficiency between the two variants of the

protein. Therefore, to study the *in vivo* relevance of these results, danofloxacin was administered to 6 homozygous non-carrier cows of the polymorphism (Y/Y) and 6 heterozygous cows for the Y581S polymorphism (Y/S) at a dose of 1.25 mg/kg and plasma and milk levels of this antimicrobial were analyzed (article I). The results obtained in plasma were very similar to those obtained by Shem-Tov et al. (1998) and Shojaee Aliabadi and Lees (2003). No differences were reported in plasma AUCs between the two genotypes (1.59 ± 0.29 $\mu\text{g}\cdot\text{h}/\text{ml}$ in Y/Y cows versus 1.44 ± 0.12 $\mu\text{g}\cdot\text{h}/\text{ml}$ in Y/S cows) (Figure 1, table 1, article I). Although, ABCG2 has the capacity to affect plasma levels of its substrates (Keskitalo et al., 2009; Kim et al., 2007), different studies have shown that for certain substrates the plasma profile is unaffected even in those cases where the transporter is absent as shown for Abcg2^(-/-) mice (Vlaming et al., 2009). Furthermore, our previous studies using Abcg2^(-/-) mouse models and sheep have shown that plasma concentrations of danofloxacin are not affected by this transporter in these species (Real et al., 2011a). Even human Q141K SNP does not affect the plasma disposition of all ABCG2 substrates (Kim et al., 2007; Adkison et al., 2008; Keskitalo et al., 2009). This effect might respond to the presence of other transporters involved in plasma availability of these molecules hiding a potential effect caused by ABCG2. However, a lack of any effect from Y581S SNP on the plasma disposition of danofloxacin is a positive outcome for the therapeutics of systemic infections, since a change in plasma levels of the antibiotic would affect treatment efficacy as a result of the concentration-dependent effect of fluoroquinolones.

Regarding to milk, the results are consistent with those obtained by Shem-Tov et al. (1998) but only for Y/Y cows. Danofloxacin levels found in Y/S cows were significantly higher than those detected in Y/Y cows at 5 and 11 h (Figure 2, article I). The AUC milk/plasma ratios indicate that Y/S cows transfer two-fold more drug from plasma into milk (4.09 ± 1.07 in Y/Y cows versus 8.81 ± 3.69 in Y/S cows). In addition, the elimination parameters, mean residence time (MRT) and elimination half-life ($T_{1/2}$),

were significantly lower in Y/S cows (Table 1, article I). Fluoroquinolones are rapidly eliminated from the body. The withdrawal period for this dose is only 48 hours, which might hide a greater effect on mean residence time (MRT) and half-life. However, it cannot be discarded that for drugs with longer withdrawal periods, Y581S polymorphism may have a stronger effect and show differences between the two variants in critical parameters for dairy industry as the MRT. Anyway, the spectacular results obtained in the heterozygous cows with regard to their enhanced ability to secrete this antimicrobial to milk are consistent with previous studies that had been performed *in vitro* by our group, validating the suitability and predictive value of our *in vitro* models.

After the positive results obtained with this substrate we decided to perform a new experiment with a new formulation of danofloxacin, Advocin 160® (6 mg/kg), which reaches higher concentrations in plasma, increasing the safety and treatment success and is also recommended for mastitis treatment. Due to the specific role of ABCG2 in drug concentration in the mammary gland, the implications of a potential effect of the polymorphism Y581S are particularly relevant in this case. Our results indicate that the plasma levels obtained in this study were similar to those obtained by Mestorino et al., (2009). Again, no significant differences were obtained in plasma levels although a tendency was noted for plasma with lower AUCs in Y/S cows ($7.88 \pm 2.21 \mu\text{g}\cdot\text{h}/\text{ml}$) compared to Y/Y cows ($10.83 \pm 3.38 \mu\text{g}\cdot\text{h}/\text{ml}$) even though these differences did not reach significance ($p=0.16$) (Figure 2, table 1, article II).

With regard to the concentrations of danofloxacin in milk, the levels obtained in our study were between two and three times higher than those obtained in other studies. These discrepancies can be attributed to differences in experimental design or, physiological conditions of the animals whose weight and milk production were much higher than those used in the study conducted by Mestorino et al. (2009). In addition, the composition of the diet is another major factor that can dramatically affect the

availability of ABCG2 substrates as reported for natural modulators obtained from soy. Diets enriched in flavonoid precursors may affect milk secretion of ABCG2 substrates (Pulido et al., 2006; Perez et al., 2013). With regard to the polymorphism-driven differences in milk, our analysis showed that the AUCs for milk were 50% higher for Y/S cows compared with Y/Y cows (Figure 3, article II). This effect is more moderate than that obtained using a dose 1.25 mg/kg, which illustrates the relevance and necessity to perform specific studies for each dose as the results obtained might not be easily predictable. However, as a result of lower levels of drug in the plasma of Y/S individuals, if we consider the AUCs milk/plasma ratio, the capacity to transfer danofloxacin to milk remains two-fold higher for the Y/S animals (3.42 ± 1.32 cows Y/Y versus 7.02 ± 3.12 in cows Y/S (Table I, article II)). These results confirmed that the polymorphism Y581S increases the secretion into milk of danofloxacin with particularly relevant implications for the treatment of mastitis because of the observed differences in milk concentration depending on the genotype.

Enrofloxacin is another drug that is widely administered in cattle. The administration of this drug results in its rapid metabolization to ciprofloxacin, the major active metabolite generated. Prior to study the pharmacokinetic behavior of enrofloxacin and its metabolite ciprofloxacin in relation to bovine Y581S polymorphism, an *in vitro* study was conducted to evaluate the potential impact of this polymorphism *in vivo*. Enrofloxacin was previously tested by Real et al., (2011b), which showed that cells overexpressing the S581 variant transport enrofloxacin much more efficiently than cells transduced with the Y581 variant similarly to the transport of the antimicrobial danofloxacin. To complete the *in vitro* study, we studied transepithelial transport of ciprofloxacin, to determine a differential transport capacity between the two variants, as its precursor enrofloxacin. The results showed that ciprofloxacin was a substrate of the bovine ABCG2 and that the S581 variant transported more efficiently the compound than the Y581 variant (Figure 2, article III).

To evaluate *in vivo* whether the ABCG2 transporter was involved in the secretion of enrofloxacin into milk, an experiment with lactating wild-type and Abcg2^(-/-) mice was carried out demonstrating that ABCG2 was involved in the secretion into milk of this drug. Enrofloxacin milk secretion in Abcg2^(-/-) mice was significantly lower than in wild-type mice (2.23 ± 0.20 mg/ml in wild-type mice versus 1.74 ± 0.26 mg/ml in Abcg2^(-/-) mice) (Figure 1, article III). The role of Abcg2 in the secretion of ciprofloxacin into milk was previously studied and demonstrated by Merino et al. (2006). These studies highlight the interaction and effect of the ABCG2 transporter in the secretion into milk of enrofloxacin and ciprofloxacin.

With these preliminary results obtained in the lab, commercial enrofloxacin (Baytril® 10%) was the next interesting candidate to study in farm for the potential effect of the bovine SNP on its secretion into milk and plasma distribution. The experimental design consisted in administering Baytril® 10% at a dose 2.5 mg/kg to five Y/S cows and six Y/Y cows. Plasma pharmacokinetic parameters obtained (Table 1, article III) were similar to those obtained for the same dose by McKellar et al. (1999).

Plasma analyses showed that AUCs were significantly lower for Y/S genotype carriers (1.86 ± 0.32 µg·h/ml) compared to Y/Y genotype carriers (2.33 ± 0.23 µg·h/ml) (Table 1, article III). Although in our previous studies a tendency was observed, this is the first study showing an effect of the bovine Y581S polymorphism on plasma distribution of a substrate. ABCG2 is typically involved in the absorption and systemic secretion of drugs. Therefore, due to the greater activity of the polymorphic variant, the Y581S polymorphism could promote a more rapid and efficient systemic elimination of its substrates in the urine (main route of elimination of enrofloxacin (Martinez et al., 2006)), bile, intestinal lumen and milk. In the case of ciprofloxacin, plasma levels were also lower for Y/S cows (Table 1, article III) but in this case this outcome cannot be only attributed to the effect of the polymorphism, but also in part to the different initial concentrations of the parental compound enrofloxacin. In fact, differences in plasma

AUCs for ciprofloxacin between the two groups were similar to that of enrofloxacin, around 20%.

With respect to differences in the pharmacokinetic parameters obtained in milk we also observed an effect associated to the genetic variants of the transporter. Similarly to previous studies with danofloxacin, AUC₀ and AUC milk/plasma ratio values were significantly higher in Y/S animals compared to Y/Y animals (Table 2, article III). AUCs milk/plasma ratio were 1.5 times higher for enrofloxacin (0.52 ± 0.03 in Y/Y cows versus 0.84 ± 0.15 in Y/S cows) and 2.5 times higher for ciprofloxacin in Y/S cows compared to Y/Y cows (3.00 ± 1.19 in Y/Y cows versus 7.79 ± 0.66 in Y/S cows). These differences were similar to those obtained in previous studies with danofloxacin.

On the other hand, AUC milk/plasma ratio values for ciprofloxacin were up to 9 times greater than those of enrofloxacin (Table 2, Article III), which indicates that ciprofloxacin is much more efficiently accumulated in milk than enrofloxacin. Regardless of genotype, milk therapeutic profile is dominated by ciprofloxacin in milk (Figure 4, article III). These differences in milk concentrations between enrofloxacin and ciprofloxacin had been previously observed by other authors (Kaartinen et al., 1995; Rantala et al., 2002; Idowu et al., 2010). These differences might occur because part of enrofloxacin can be metabolized to ciprofloxacin in the udder. It is also possible that the low pH of the milk ionizes ciprofloxacin and consequently this molecule would be trapped in milk (Malbe et al., 1996). Due to the partial metabolization of enrofloxacin to ciprofloxacin in the udder, we cannot rule out that some of the genotype-driven differences in the concentrations of ciprofloxacin are caused by differences in the concentrations of enrofloxacin. However, the difference between genotypes in the AUC milk/plasma ratios for enrofloxacin was 1.5 times higher for Y/S cows, while for ciprofloxacin was 2.5 higher for Y/S cows. This indicates the occurrence of an additional effect produced by the differences in the genotype of cows.

Some studies have suggested that polymorphisms can affect expression of certain transporters even for heterozygous individuals (Wojnowski and Brockmöller, 2004; Visser et al., 2014). To determine whether the obtained differences are due to an increased activity of the transporter or to an increased expression of the protein, a study of RNA and protein expression by qRT-PCR and western blot, respectively, in the two groups of animals was carried out. Based on the results derived from the study of RNA expression of both genetic variants it was not possible to reach conclusions due to the high variability of the results (Figure 4a, article II). However, protein expression studies of the plasma membrane of mammary alveolar cells (Figure 4b, article II) showed very similar expression level in both groups, indicating that the polymorphism Y581S does not induce the expression of the transporter. Similarly to the *in vitro* outcome, these results showed *in vivo* for the first time that the Y581S SNP is a gain-of-function polymorphism that confers a greater capacity to the bovine ABCG2 to transport its substrates.

Defining and studying the factors involved in drug concentration in milk are essential to more accurately predict the unexpected contamination of dairy products and treatment failure. These results can help to identify those factors that are relevant for interindividual variability in response to drug treatment, and might help to adjust the used drug doses and withdrawal periods. Since the fluoroquinolones studied have a dose-dependent effect, it is possible that specific tissues with high ABCG2 expression might be specifically affected by the polymorphism and therefore, not reaching the minimum inhibitory concentration, promoting the development of resistances (Illambas et al., 2013). Moreover, these differences in transport due to polymorphism, are not exclusive for antibiotics; other toxins recognized by ABCG2 present in the body and derived from the diet might also be affected by the polymorphism and differentially concentrated in milk. Among them we find the fungal toxin aflatoxin B1, or the phototoxin pheophorbide (Wassermann et al., 2013b).

8.1.2. Effect of the Y581S polymorphism on the physicochemical properties of milk and metabolomic analysis of plasma, milk and urine

In the previous section we have shown a drastic effect of Y581S bovine polymorphism of ABCG2 on the distribution of xenobiotics but the role of ABCG2 is not limited to interaction with toxins and exogenous compounds. In humans the role of ABCG2 in the secretion of endogenous compounds into milk has been extensively studied. In 2007 van Herwaarden et al., showed that vitamin B₂ was transported to milk by ABCG2. Other papers studied the relation between human polymorphisms of ABCG2 and distribution of vitamin K₃ and uric acid (Jani and Krajcsi, 2014). Some of these endogenous compounds such as uric acid and vitamins have antioxidant activities and might affect the physicochemical properties of milk and its further industrial processing. Analyzing the physicochemical properties of milk can provide important information of the effect of the polymorphism on the quality of milk. To complete the characterization of this polymorphism *in vivo* a study of the physicochemical properties of milk and a metabolomic study of plasma, milk and urine of cows carriers and non-carriers of SNP Y581S was performed to evaluate differences in the properties of milk in both groups.

From the parameters studied to analyze the physicochemical properties of milk, the statistical analysis revealed that the conductivity was around 20% higher in Y/S cows compared to Y/Y cows (Table 1, annex I). High levels of conductivity are related to premastitic stages because of the increase of intracellular ions in milk (Diaz et al., 2012; Gargouri et al., 2014.). Furthermore, it is reported that the conductivity is a sensitive parameter to multiple variables and its increase in milk may be caused by a reduction of the content of oxidizing agents in milk such as hydrogen peroxide (Sakai et al., 2010). As it will be discussed later, polymorphism-carrier cows can secrete certain antioxidants into milk more efficiently than Y/Y cows, which might reduce the

concentration of some superoxide agents like the hydrogen peroxide and therefore increase the conductivity observed in these cows.

The redox potential is a parameter with relevant impact on the organoleptic and microbiological properties of dairy products (Tachon et al., 2010). Although in this study, no significant differences were found in the redox potential, a tendency to be higher in the milk of Y/S cows was observed ($p = 0.10$) (Table 1, Annex I) which may also be due to the differential presence of certain antioxidants like uric acid as will be discussed later in Article III.

The lactose content was significantly higher in milk from Y/Y cows compared to the milk produced by Y/S cows (Table 1, annex I). It is known that bacterial ABC transporters can transport disaccharides such as maltose (Bordignon et al., 2010). In mammals, however, there is no evidence that ABC transporters can transport carbohydrates such as lactose so far, so how the Y581S polymorphism can affect the levels of lactose in milk production remains unclear.

The comparative study of protein and fat content between the two groups of cows showed no difference between them, which disagrees with that reported by Cohen-Zinder et al. (2005). Since both parameters are closely related to diet, the differences with our results may be due to an effect of the possible different forage supplied to the animals and other animal management features such as the number of milkings per day, which drastically affect milk properties. Furthermore, the number of individuals could have limited our study of the physicochemical properties of milk.

The metabolomic studies carried out by the research groups of CEBAS (Murcia, Spain) and NKI (Amsterdam, The Netherlands), obtained similar findings. In both cases we observed a greater accumulation of certain metabolites in the milk of Y/S cows compared to Y/Y cows (Figures 7 and 9, annex I). It was also observed that some metabolites were found in lower concentration in the plasma of Y/S cows (Figures 7

and 8, annex I). In general these results are similar to those obtained in our studies with fluoroquinolones. As a consequence of the higher activity of the Y581S variant of the transporter, the cows carriers of the polymorphism secrete some metabolites more efficiently into milk, and at the same time some metabolites are more efficiently cleared from the system, and are found in lower concentration in the plasma of these cows. This effect is confirmed in the metabolomic study of urine, where from metabolites with significant differences between both sets of cows (Figure 10, annex I), 90% were concentrated more efficiently in the urine of the Y/S cows.

Due to the special interest of the dairy industry for the components of milk, from all the hits obtained in milk, one matched with databases as uric acid, corroborating the different concentration in milk of both set of cows observed in article IV. From the rest of metabolites with different concentration between the two groups of cows, two of them could be identified by mass spectrometry. One of these candidates that is transported more efficiently by the polymorphic animals was the hippuric acid (Figure 11, annex I). This metabolite is formed as a result of the combination of benzoic acid and glycine. The identification of this molecule as a potential substrate of the bovine isoform of ABCG2 is supported by previous studies that have shown that this compound, at physiological concentrations, can inhibit the estrone sulfate transport mediated by ABCG2. Since this compound is also found in vegetables and fruits (Mutsaers et al., 2011), diets enriched in hippuric acid could be used to control the secretion of other substrates of ABCG2 to milk. On the other hand, direct ABCG2 transport of this molecule has an interesting potential in ruminants because an increased urinary excretion of hippuric acid can inhibit N₂O emissions in cattle. Hippuric acid secretion removes the nitrogen potentially metabolizable by bacteria in the digestive tract of ruminants. Given the environmental problem of the intensive exploitation of livestock that contributes to the greenhouse effect for the production of noxious gases, it is interesting to understand the factors that can affect harmful gas

emissions for the environment. However, it is still necessary to further study how hippuric acid reduces N₂O emissions since the process is not fully understood yet (Dijkstra et al., 2013). Moreover, in our metabolomic analysis of urine we could not determine the concentrations of hippuric acid so it is necessary to perform a specific study analyzing the content of this compound in urine to better understand the biological consequences of this finding.

The other molecule that was identified is the N-cinnamoylglycine. Similarly to the hippuric acid, this compound is an intermediate that is conjugated with glycine for the removal of phenylpropanoic acid (Rouge et al., 2013). The recognition of this molecule by ABCG2 may respond to a strategy of the organism to control the toxicity caused by aromatic compounds through their elimination as N-cinnamoylglycine. During the preliminary identification of compounds it was noted that the potential identity of many hits were glycine-conjugated compounds. Since this transporter has a high affinity for phase II conjugated metabolites with sulfates (van de Wetering and Saptho, 2012) and glucuronides (Alvarez et al., 2011), this mechanism could be extended to those molecules which are conjugated with glycine as a cellular mechanism for recognition and elimination of some metabolites. However, to confirm the direct relationship of these two molecules with ABCG2 further *in vitro* studies are necessary with our model of MDCK-II cells to corroborate first, the existence of a direct transport mediated by ABCG2 and second, differences in the transport ratio in a genotype-dependent manner.

8.1.3. Influence of the polymorphism in the secretion into milk of compounds with nutritional benefits

Inside the group of molecules that have been described as substrates of the murine and human isoform of ABCG2 there are some naturally occurring substances whose relevance is key to the dairy industry as uric acid or riboflavin (vitamin B₂). In our metabolomic study it was possible to identify uric acid (m/z 167.021) as one of the hits

with significant differences in the milk of the two groups of cows. However, in this study riboflavin or enterolactone showed no differences probably because the set parameters to select the differential compounds were too restrictive and because the endogenous levels of these compounds are very low and would require specific extraction for a more reliable and rigorous study. Given its potential relevance to the industry, a detailed study of these three compounds was conducted.

The antioxidant potential of the uric acid found in milk is similar to that of carotenoids, ascorbic acid and α -tocopherol, conferring to this purine an important role for milk quality and for its antioxidant properties (Larsen and Moyes, 2010). It contributes to the redox potential of milk (as observed in the study of the physicochemical properties of milk), although this parameter is also dependent on other molecules such as ascorbic acid and α -tocopherol (Aycicek et al., 2006).

The diet received by the cows is another factor that determines the nutritional capacity of milk. Lignans are polyphenols that can be found in almost all species of the plant kingdom. Flax (*Linum usitatissimum*) is the most abundant source of lignans with high amounts of the precursor secoisolariciresinol diglucoside (SDG) (Axelson et al., 1982). Enterolactone is a metabolite derived from secoisolariciresinol lignan produced by the ruminal and intestinal microbiota, which has been found in significant concentrations in milk (Antignac et al., 2004). When cows are fed with flaxseed and *Trifolium repens* they accumulate high concentrations of enterolactone in milk (Steinshamn et al., 2008; Petit et al., 2009). Enterolactone possesses antioxidant activity and dietary intake of lignans and their derivatives, as part of a healthy diet, has been associated with protection against cardiovascular disease, diabetes and metabolic syndrome, cancer, oxidative stress and inflammation (Adolphe et al., 2010). It has been published that the intake of lignans and derivatives in the diet protects against certain chronic diseases (Adolphe et al., 2010).

Finally, van Herwaarden et al. (2007) has also demonstrated the role of ABCG2 in milk secretion of riboflavin with an obvious interest and nutritional impact for the dairy industry.

Therefore, we decided to study in detail the effect of bovine Y581S polymorphism of ABCG2 on milk secretion of these compounds: uric acid, riboflavin and enterolactone. Our MDCK-II models overexpressing the Y581 and S581 variants of the bovine ABCG2 have been demonstrated to be adequate to predict milk secretion of compounds and to understand factors underlying interindividual differences. However, at the moment, the effect of the alteration of the concentration of specific compounds in milk quality cannot be precisely predicted. This is the case for uric acid, a purine metabolite that can act as a potent antioxidant. Our *in vitro* studies confirmed that uric acid is a bovine ABCG2 substrate (Table 2, article IV). Furthermore, the *in vivo* results concluded that uric acid concentration was greater in the milk of Y/S cows than Y/Y cows ($421.1 \pm 54.2 \mu\text{M}$ for Y/S cows *versus* $254.4 \pm 26.3 \mu\text{M}$ for Y/Y cows (Figure 3, article IV)). These studies show that ABCG2 transports uric acid into milk and that the concentration of uric acid is higher in the milk of Y/S cows. Since physiological concentrations of uric acid have been shown to inhibit the transporter (Dankers et al., 2013), this factor should be considered in the study of differential milk secretion of specific compounds.

Riboflavin has also been tested in our *in vitro* model for its relevance as a micronutrient in several biological processes. Previous studies demonstrated that this vitamin is substrate of the human and murine isoform of the transporter (van Herwaarden et al., 2007). These authors further found that this transporter limited bioavailability of riboflavin in plasma and organs and this vitamin was also actively secreted into milk by this protein. However, the relevance of this finding for the veterinary field is unknown. Transepithelial transport experiments in polarized MDCK-II cells identified riboflavin as a bovine ABCG2 substrate. These results also showed the

presence of a basolateral transport mechanism for riboflavin, which is in agreement with previous studies, which suggest the existence of specific transporters of riboflavin in the basolateral membrane of cells (Yonezawa and Inui, 2013). However, our studies showed the presence of another transport mechanism mediated by the bovine ABCG2 in the apical membrane. According to our results *in vitro* with MDCK-II cells, the S581 variant is more active in the transport of riboflavin because it can reverse the basolateral transport of riboflavin more efficiently (Figure 4, article IV).

The relevance of this transporter in the concentration of this vitamin in milk (at least in a lab environment) is questionable because of the lack of effect in the offspring of Abcg2^(-/-) female mice fed with a diet deficient in riboflavin. In any case, the study of the factors that could affect the vitamin composition of milk is of great interest to the dairy industry. The analysis of milk from the cows carriers and non-carriers of the polymorphism revealed no differences between the two experimental groups, probably because the effect on cells overexpressing the transporter is drastic and stronger than the effect observed in a more complex and realistic scenario such as a farm with heterozygous individuals. In addition, the cofactors FAD and FMN which are precursors of vitamin can mask a difference in the accumulation of the vitamin (van Herwaarden et al., 2007).

Regarding to the interaction of the bovine ABCG2 with enterolactone, in our study the levels in plasma and milk of cows were comparable to the concentrations obtained in other studies using these ruminants (Antignac et al., 2004; Gagnon et al., 2009). Enterolactone is considered another factor that may promote an antioxidant environment in the mammary gland (Cortes et al., 2012; Schogor et al., 2013.). Our *in vitro* studies have shown that enterolactone is a potent and specific bovine ABCG2 substrate and also that is more actively transported by S581 variant of the bovine ABCG2 (Table I, article IV). In ruminants, lignans are metabolized in the rumen and are available for absorption in the small intestine as enterolignans (Petit et al., 2009).

Njåstad et al., (2014) studies suggested that the concentration of enterolactone in milk may be limited by rejugation processes and secretion in the rumen wall. Recent studies located ABCG2 in bovine rumen, intestine and liver (Lindner et al., 2013; Haslam and Simmons, 2014) so it is not surprising that ABCG2 could take part in the milk secretion and at the same time limit the systemic disposition of this molecule by rejugating enterolactone with sulfate or glucuronide moieties as has been demonstrated for isoflavones, natural substrates of the transporter derived from soy intake (Alvarez et al., 2010). Plasma levels of enterolactone obtained in Y/S cows were much lower than in Y/Y cows (Figure 2, Article IV), indicating the important role of this polymorphism in the plasma availability of enterolactone. The milk/plasma ratio was much higher in Y/S cows than in the Y/Y cows (2.99 ± 0.46 for Y/S cows versus 1.44 ± 0.50 in Y/Y cows) confirming that the SNP acts as gain-of-function polymorphism.

Apart from being an ABCG2 substrate, enterolactone shows the capacity to inhibit the transporter. *In vitro* experiments with enterolactone 100 and 200 μM can reverse the accumulation of mitoxantrone up to 70% (Figure 1, article IV). The S581 variant showed lower inhibition of transport of mitoxantrone probably because this variant has a greater transport capacity and thus it is harder to inhibit the transporter. The same effect has been described in previous studies with other inhibitors such as selamectin or triclabendazole sulfoxide and sulfone (Merino et al., 2009; Barrera et al., 2013).

8.2. Enterolactone as substrate and modulator of ABCG2

Dietary sources of plant lignans are flax grains, seeds, fruits and vegetables, olive oil, and beverages such as tea, coffee, and wine (Kuijsten et al., 2005). In general, under normal dietary conditions, enterolactone is the enterolignan that reaches the highest concentration in plasma (Kuijsten et al., 2006) and is the most effective metabolite in the inhibition of cell proliferation (Corsini et al., 2010; Azrad et al., 2013). Owing to the potential beneficial effects of enterolactone and the complex relationship between the colonic environment and other factors contributing to systemic exposure of

such compounds to ABC transporters, a study of the interaction of ABCG2 with enterolactone could be of particular relevance. In this regard, the aim in this section was to assess the potential role of enterolactone as an ABCG2 substrate and/or inhibitor in mice and sheep using MDCK-II epithelial cells transduced with the transporter.

8.2.1. Cell culture and Abcg2^(-/-) mice studies with enterolactone

After the excellent results obtained with enterolactone and the bovine ABCG2, we studied its interaction with murine isoform (as a preclinical model) and the human ABCG2. Furthermore, the specific *in vivo* role of the ABCG2 transporter in the disposition of this mammalian lignan was evaluated using wild-type and Abcg2^(-/-) knockout female mice by determining the endogenous enterolactone milk/plasma ratio found in the two types of mice (article V). The interest in these compounds has increased in recent years as part of research related to the effects of the activity of specific nutrients in the prevention and control of cancer (Adlercreutz, 2002; Del Rio et al., 2013). Enterolactone and enterodiol are considered biologically the most active enterolignans. Because of these health beneficial properties, several products based on flax seed hulls, which are mainly used as food supplements, have appeared on the market. It has not been shown a direct interaction of enterolactone and human and murine ABCG2 transporters yet. Our studies were performed using MDCK-II transduced cells and wild-type and Abcg2^(-/-) mice to elucidate the role of ABCG2 in the bioavailability of enterolactone and study the role of ABCG2 in enterolactone milk transfer.

The results of our study showed a direct involvement of ABCG2 on the transport of enterolactone. Transepithelial transport experiments using parental MDCK-II cells and MDCK-II cells transduced with murine and human transporter showed that enterolactone was apically transported in cells overexpressing the transporter with a transport basal-apical/apical-basal ratio of 16.5 and 3.1 for murine and human isoforms

respectively. This transport was reversed with the specific inhibitor of ABCG2 Ko143 (Figure 1, article V), confirming *in vitro* the specific role of ABCG2 in the transport of enterolactone. Previous studies have shown that at concentrations in the μ molar range, enterolactone can modulate different components of intracellular signalling cascades of growth, proliferation or apoptosis (Crozier et al., 2009; Corsini et al., 2010). Therefore our findings may have important physiological significance in those organs with high expression of the transporter as the intestine, the syncytiotrophoblast and mammary gland (Mao and Unadkat, 2015).

To confirm the relevance of these *in vivo* results, endogenous plasma and milk enterolactone levels were analyzed in mice, demonstrating the role of ABCG2 in the milk secretion of this compound. The milk/plasma ratio of this compound in *Abcg2*^(-/-) mice was much lower than that of wild-type mice (0.4 ± 0.1 versus 6.4 ± 2.6) (Table 1, article V), which indicates that the *Abcg2*^(-/-) mice lose the capacity to concentrate this metabolite in milk up to 90% compared to wild-type mice. The mean plasma concentrations in mice were similar to those obtained in humans (57 ± 13 nM) (Kuijsten et al., 2005; Adolphe et al., 2010) (Table 1, article V).

The distribution of the conjugated forms of lignans has been previously shown to be dependent on the ABCG2 and ABCC2 transporters (Lampe et al., 2006; Krumpochova et al., 2012; van Wetering and Sapth, 2012). They showed that enterolactone sulfate is a low affinity substrate of ABCG2 and that the *Abcg2*^(-/-) mice show altered levels of enterolactone sulfate, demonstrating the relevance of this transporter in the general disposition of not only enterolactone but also its derivatives. Controlled feeding studies have shown that lignan excretion by ruminant in urine and milk is dose-dependent (Lampe et al., 2006; Petit et al., 2009), thus the interaction of enterolactone and its conjugates with ABCG2 and intestinal microbiota are essential factors affecting the overall distribution of enterolactone.

To complete the characterization of enterolactone, the capacity of this compound to modulate the activity of the murine and human ABCG2 was studied. Mitoxantrone accumulation studies using flow cytometry with parental MDCK-II cells and transduced with murine and human ABCG2 showed that enterolactone inhibits the transport of mitoxantrone. Enterolactone 200 μ M increased the intracellular accumulation of this compound by 38% (Figure 2, article V). Since the physiological concentration of enterolactone range from 10 to 1000 μ molar in the lumen of the colon (Kitts et al., 1999) and since according to our experiments these concentrations inhibit transporter activity, a possible exposure to diets rich in lignans may have physiological implications due to interactions with other substrates. Furthermore, these results may be of interest in therapies that aim to reverse resistance mediated by ABCG2 to chemotherapy treatments (Allen et al., 2002; Robey et al., 2009).

8.2.2. Modulation of the ovine ABCG2 transporter with flaxseed-enriched diets: impact on danofloxacin milk secretion

The interactions between conventional drugs and certain natural products derived from plants are increasingly common in clinical practice. To evaluate these interactions, the techniques traditionally used to study drug-drug interactions can be used to study these new mechanisms (Brantley et al., 2014).

Recently, many studies have focused on the beneficial effects of supplementation of flaxseed in ruminant diets, and how these diets enhance the presence of metabolites derived from SDG in biological fluids (Antignac et al., 2004 ; Cortes et al., 2013; Schogor et al., 2014). Flaxseed and their derivatives are considered an acceptable source of energy and protein (Schogor et al., 2014). Enterolactone is mainly produced in the rumen by *Prevotella spp.* that biotransforms SDG and thus promotes its absorption (Schogor et al., 2014). After ingestion, the SDG is biotransformed in secoisolariciresinol (SECO). Then, the ruminal and intestinal microbiota transforms SECO in enterolactone and enterodiol (Petit et al., 2009). Therefore, in this case we

considered necessary to study drug-food interactions with SDG derivatives since they may affect the pharmacokinetics and milk secretion of certain compounds in sheep (article VI). This type of interactions have already been described for other compounds derived from the diet as isoflavones (Pulido et al., 2006; Vlaming et al., 2009; Perez et al., 2013). Previous studies with enrofloxacin and danofloxacin showed that diets rich in soy act on ABCG2 transporter activity in sheep and reduce the secretion of these compounds to milk by more than 30% (Pulido et al., 2006; Perez et al., 2013).

To test the potential drug interaction involving enterolactone, a transepithelial transport study of danofloxacin with enterolactone was performed, which showed that enterolactone is an efficient inhibitor of danofloxacin transport mediated by ABCG2. Enterolactone 100 μ M reversed almost completely danofloxacin transport mediated by ovine ABCG2. Our results showed that the basal-apical/apical-basal transport ratio was reduced from 9.22 ± 0.69 to 1.36 ± 0.11 in the presence of enterolactone, which was similar to that obtained with the parental cells (Figure 1, article VI).

Furthermore, a study was carried out with sheep which were fed with diets enriched with flaxseed to test this drug interaction *in vivo*. Diet supplementation with the precursor of mammalian lignans SDG increased milk and plasma accumulation of enterodiol, enterolactone and their derivatives. There are multiple studies that have assessed the amount of enterolactone and enterodiol in plasma and cow's milk after dietary supplementation with lignans (Cortes et al., 2013). Conversely, this is the first time that these parameters are measured in sheep. Similarly to cattle (Gagnon et al., 2009), we demonstrate a correlation between the amount of lignans in the diet and the amount of enterolactone in plasma and milk after diet supplementation in sheep for two weeks (Figure 2, article VI).

After supplementation of the diets with 10% and 15% for 15 days, danofloxacin 1.25 mg/kg was administered intramuscularly to animals. The obtained plasma profiles were similar to those obtained by Aliabadi et al., (2003) and Barrera et al. (2013) and

no differences were observed between the three groups of animals. With respect to the results of danofloxacin milk secretion, the results in the control group were similar to those obtained by Barrera et al. (2013) and Perez et al. (2013). In sheep that received dietary lignans, danofloxacin secretion was reduced by 25-30% (Figure 4, Table 3, article VI), which suggests the occurrence of competition process between danofloxacin and enterolactone for ABCG2 transporter in the mammary gland as observed *in vitro*. Our results showed no differences between diets enriched with 10 and 15% of lignans, probably because the high plasma concentrations of enterolactone saturate its transport into milk mediated by ABCG2. Although different enterolactone plasma concentrations were reported in both groups, the similar concentrations of danofloxacin obtained in milk from the 10% and 15% lignan supplemented sheep strengthen the hypothesis of saturation of enterolactone transport into milk (Figure 2, article VI).

We cannot exclude that other derivatives of lignans such as enterodiol (also detected in plasma and sheep milk (Figure 2, article VI)), and conjugates of enterodiol and enterolactone have an additional effect on the modulation of danofloxacin transport mediated by ABCG2. In fact, the conjugation of enterolactone with glucuronide and sulfate in the liver has been described (Gagnon et al., 2009). However, enterolactone is the most abundant lignan in cow milk after flaxseed enrichment of diet (Antignac et al., 2004) so it is likely that any effect resulting from the action of lignans on ABCG2 transporter is dominated by enterolactone although we cannot discard other modulators obtained from flaxseed. However, to understand the extent of the effect of all compounds derived from flax intake on ABCG2, further studies are needed.

Diets rich in flaxseed reported a number of nutrients and antioxidants properties to milk through enterolactone, its major metabolite. In addition to the added value of the product for its benefits, its ability to inhibit ABCG2 transporter has to be considered as

a more suitable alternative to the inhibition of milk transport of drugs with xenobiotics that usually produce a number of undesirable side effects (Mao and Unadkat, 2015).

9. CONCLUSIONES

De los resultados obtenidos en los trabajos presentados podemos obtener las siguientes conclusiones:

CONCLUSIÓN PRIMERA: Los estudios farmacocinéticos con danofloxacino en vacas Holstein portadoras y no portadoras del polimorfismo bovino Y581S a las dosis de 1,25 mg/kg y 6 mg/kg demostraron que en las vacas portadoras del polimorfismo se incrementa la secreción de danofloxacino a leche con respecto a las vacas no portadoras ya que los ratios AUCs leche/plasma aumentaron de 4,09 a 8,81 en la dosis de 1,25 mg/kg y de 3,42 a 7,02 en la dosis de 6 mg/kg.

CONCLUSIÓN SEGUNDA: Los estudios farmacocinéticos con enrofloxacino sobre vacas Holstein portadoras y no portadoras del polimorfismo bovino Y581S demostraron que en las vacas portadoras del polimorfismo aumenta la secreción de enrofloxacino a leche con unos valores de los ratios AUCs leche/plasma de 0,52 en vacas Y/Y y 0,84 en vacas Y/S. Por otra parte la secreción de ciprofloxacino a leche también aumenta en las vacas portadoras del polimorfismo con unos valores de los ratios AUCs leche/plasma de 3,00 en vacas Y/Y frente a 7,79 en vacas Y/S. Además se observó que el polimorfismo reduce la disponibilidad plasmática de ambos fármacos en torno a un 20%.

CONCLUSIÓN TERCERA: Los estudios de expresión proteica de ABCG2 bovino en función del polimorfismo a través de *western blot* revelaron que el polimorfismo bovino Y581S no afecta a la expresión de proteína en las células alveolares mamarias por lo que teniendo en cuenta nuestros resultados, puede ser considerado como un polimorfismo de ganancia de función.

CONCLUSIÓN CUARTA: El polimorfismo bovino Y581S de ABCG2 tiene efectos específicos sobre propiedades fisicoquímicas de la leche. También tiene efectos generales sobre la metabolómica de las vacas portadoras del polimorfismo

aumentando la concentración de ciertos metabolitos en leche y orina y reduciéndolos en plasma.

CONCLUSIÓN QUINTA: La enterolactona, la riboflavina y el ácido úrico son sustratos del transportador ABCG2 bovino y son transportados de manera diferencial en función del polimorfismo Y581S *in vivo* y/o *in vitro*.

CONCLUSIÓN SEXTA: La enterolactona es un sustrato y un inhibidor *in vitro* de la isoforma humana y de ratón del transportador ABCG2. Los estudios *in vivo* con ratones *wild-type* y *Abcg2*^(-/-) demostraron que ABCG2 es el principal responsable de la secreción de enterolactona a leche.

CONCLUSIÓN SÉPTIMA: La enterolactona es un eficiente inhibidor *in vitro* del transporte transepitelial de danofloxacino mediado por ABCG2 ovino, con ratios de transporte basal-apical/apical-basal de 9,22 en las células controles que sobreexpresan el transportador frente a ratios de 1,36 en presencia de enterolactona.

CONCLUSIÓN OCTAVA: Los estudios farmacocinéticos realizados en ovejas de producción lechera de raza Assaf mostraron que las dietas enriquecidas en lino un 10%, y administradas durante 15 días, disminuyen la secreción de danofloxacino a leche ya que los ratios correspondientes de AUCs leche/plasma se redujeron de 11,12 a 7,64 en los animales con la dieta.

10. CONCLUSIONS

From the results shown in this Dissertation we obtain the following conclusions:

FIRST CONCLUSION: The pharmacokinetic studies performed with danofloxacin at doses of 1.25 mg/kg and 6 mg/kg in Holstein cows carriers and non-carriers of the bovine Y581S polymorphism showed that carrier cows secrete more danofloxacin into milk since the AUCs milk/plasma ratios increased from 4.09 to 8.81 for the 1.25 mg/kg dose and from 3.42 to 7.02 for the 6 mg/kg dose.

SECOND CONCLUSION: Pharmacokinetic studies with enrofloxacin in Holstein cows carriers and non-carriers of the bovine Y581S polymorphism demonstrated that the Y581S polymorphism increases the secretion of enrofloxacin into milk since the AUCs milk/plasma ratios increased from 0.52 in Y/Y cows to 0.84 in Y/S cows. Moreover the secretion of ciprofloxacin also increased the AUCs of the milk/plasma ratios from 3.00 in Y/Y cows to 7.79 in Y/S cows. In addition, the polymorphism reduces the plasma disposition of both drugs around 20%.

THIRD CONCLUSION: Bovine ABCG2 protein expression studies with western blot in relation to the Y581S polymorphism showed that it does not affect protein expression levels in mammary alveolar cells so according to our results it can be considered a gain-of-function polymorphism.

FOURTH CONCLUSION: The Y581S polymorphism of bovine ABCG2 has specific effects on the physicochemical properties of milk. It also affects the metabolome of the cows carrying the polymorphism increasing the amount of particular metabolites in milk and urine and reducing them in plasma.

FIFTH CONCLUSION: Enterolactone, riboflavin and uric acid are substrates of the bovine ABCG2 transporter and are differentially transported by the Y581S polymorphism *in vivo* and/or *in vitro*.

SIXTH CONCLUSION: Enterolactone is a substrate and inhibitor of the human and murine isoform of the ABCG2 transporter. Moreover, *in vivo* studies with wild-type and

Abcg2^(-/-) demonstrated that Abcg2 is the main responsible of enterolactone accumulation in milk.

SEVENTH CONCLUSION: Enterolactone is an efficient *in vitro* inhibitor of the transepithelial transport of danofloxacin mediated by the ovine ABCG2, with basal-apical/apical-basal transport ratios of 9.22 in control cells overexpressing the transporter *versus* ratios of 1.36 in presence of enterolactone

EIGHTH CONCLUSION: Pharmacokinetic studies performed in Assaf sheep showed that 10% flaxseed-enriched diets administered during 15 days, can reduce the amount of danofloxacin secreted into milk since the AUCs milk/plasma ratios decreased from 11.12 to 7.64.

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12. CERTIFICADOS

CERTIFICADO DE REALIZACION DE UNA ESTANCIA BREVE

**PROGRAMA DE FORMACION DE PERSONAL
INVESTIGADOR DEL GOBIERNO VASCO**

EGONLABUR- Estancias cortas de becarios de investigación

CERTIFICADO DEL CENTRO RECEPTOR TRAS LA ESTANCIA BREVE
CERTIFICATE OF STAY IN THE HOST INSTITUTION

1. Becario/ Applicant:
Nombre y apellidos/ Name: Jon Andoni Otero Calzada
D.N.I./ National identity Card: 45667620-Q
Centro de aplicación de la beca/ Home Institution: Universidad de León
2. Centro en el que se ha realizado la estancia/ Host Institution:
Nombre/ Name: The Netherlands Cancer Institute
Dirección/ Address: Plesmanlaan 121 1066CX, Amsterdam
País/ Country: The Netherlands
3. Investigador responsable en el centro de la estancia/ Responsible person in the Host Institution
Institución/ Institution: The Netherlands Cancer Institute, Division of Molecular Oncology
Nombre/ Name: Koen van de Wetering
Cargo/ Position: PhD
CERTIFICO: que el becario arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde 11/02/2013 hasta 24/05/2013
THIS IS TO CERTIFY: that the above mentioned person has performed a stay in this Institution in the following dates: From: 11/02/2013 To: 24/05/2013
Firma y Sello/ Signature & Stamp
<p>The Netherlands Cancer Institute Antoni van Leeuwenhoek hospital Plesmanlaan 121 1066 CX Amsterdam The Netherlands Dr. Koen van de Wetering</p>

INFORME DE EXPERTOS INTERNACIONALES

INSTITUT PASTEUR

Paris, 28th September 2015

To whom it may concern:

The thesis entitled "Papel del transportador ABCG2/BCRP y sus polimorfismos en la secreción activa de compuestos a leche y su modulación a través de compuestos naturales en rumiantes" presented by the PhD student Jon Andoni Otero Calzada has been fully reviewed and positively considered due to both the experimental procedures involved and the excellent results achieved.

Nowadays the presence of drugs or other toxic residues in milk appears as a public health risk worldwide. The mechanisms by which these processes take place must be addressed in depth in order to avoid the appearance of allergies, bacterial resistances or to be able to market new products with improved and higher qualities. In this regard, the ATP-binding cassette transporter ABCG2/BCRP is a membrane transporter which influences the absorption, distribution and excretion of many compounds into milk when expressing in the mammary gland.

The present doctoral dissertation reviews the effect of the ABCG2 Y581S polymorphism on the secretion into milk of the fluoroquinolone danofloxacin and other widely used veterinary antibiotics: enrofloxacin and its metabolite ciprofloxacin. The impact of the secretion of other important natural compounds into milk (enterolactone, vitamin B₂ and uric acid) was also studied both *in vitro* and *in vivo*, as well as the interaction of enterolactone with human, murine and ovine isoforms of the transporter ABCG2. In addition, the physical and chemical properties of milk and the potential changes or overall effects of the presence of the polymorphism into the metabolome were also identified. These findings show the paramount importance of the presence of this polymorphism to modulate the amount of drugs and natural compounds into milk.

In my opinion, the results are very interesting and significant and the candidate has gained wide expertise and acquired a strong background, also while working on collaborative projects. For these reasons I fully support his PhD candidacy. If you have any questions please do not hesitate to contact me.

Sincerely,

Estefanía Calvo Álvarez

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INFORME DE EXPERTOS INTERNACIONALES

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October 5, 2015

To whom it may concern,

Evaluation of the PhD thesis entitled "*Papel del transportador ABCG2/BCRP y sus polimorfismos en la secrecion active de compuestos a leche y su modulacion a traves de compuestos naturales en rumiantes*" by Jon Andoni Otero Calzada.

The efflux transporter ABCG2 is expressed in key protective barriers like the intestine and blood-brain barrier, and in excreting organs like the kidneys. Therefore, it is an important player in the defense against many harmful xenobiotics. Its function in the mammary gland is dual, however. ABCG2 pumps potentially harmful xenobiotics into milk, but also enriches it with potentially beneficial metabolites like vitamins and antioxidants. This thesis describes the role of the Janus-faced transporter ABCG2 in the mammary gland of dairy cows and sheep, and mice.

In the first three chapters, Otero describes that the prevalent heterozygous Y581S gain-of-function mutation in ABCG2 increases the milk concentration of the antibiotics danofloxacin, ciprofloxacin and enrofloxacin. These findings are important for the treatment of mastitis and to reduce the amount of antibiotic residues in milk for human consumption. In the next chapter, Otero shows that the same mutation also increases milk levels of uric acid, riboflavin and enterolactone. The role of ABCG2 in the excretion of enterolactone into milk is confirmed in knockout mice in chapter 5. These chapters build up to the final chapter, in which it is shown that the excretion of danofloxacin into milk can be inhibited by coadministration of flaxseed, a rich source of enterolactone. This final chapter demonstrates that it is feasible to modulate the excretion of antibiotics into milk with relatively harmless compounds like lignans. These results are important from an economic, as well as a food safety point of view.

The thesis represents an extensive body of work that includes a wide range of *in vitro* and *in vivo* experiments that were well-designed and -conducted. Importantly, many of the chapters have already been published in international journals. Through this thesis Otero demonstrates technical competence and thorough knowledge of the subject matter. I consider the candidate ready to proceed to the oral defense of his thesis and wholeheartedly support his PhD candidacy.

Sincerely,

Robert S Jansen, PhD
Postdoctoral Associate