**Universidad Autónoma Metropolitana, Iztapalapa**

# Caracterización de los efectos de protección hepática y renal del factor de crecimiento de hepatocitos en la colestasis intrahepática

**TESIS**

Para obtener el grado de Doctora en Biología Experimental

**Presenta**

M. en Biol. Exp. Elsy Soraya Salas Silva

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Ciudad de México, 1 de Julio del 2020

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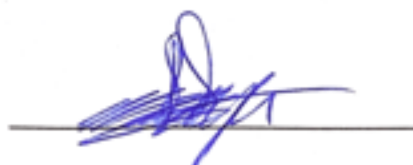

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## Resumen

La colestasis es un síndrome clínico común a un gran numero de hepatopatías, en la que la producción de bilis o su tránsito a través del tracto biliar se ve afectada debido a causas funcionales u obstructivas; la consiguiente retención intracelular de los componentes biliares tóxicos genera daños en el parénquima, en gran medida a través de mecanismos oxidantes mediados por el estrés. El factor de crecimiento de hepatocitos (HGF) y su receptor c-Met representan la primera línea de defensa contra los factores hepatotóxicos, al inducir la activación de Nrf2 que, a su vez, conduce a una respuesta antioxidante y reparadora. En este estudio, evaluamos la capacidad del HGF para contrarrestar el daño causado por el agente colestásico modelo, α-naftil isotiocianato (ANIT). HGF tuvo claros efectos anti-colestáticos, como se desprende de la mejora tanto en el flujo de bilis como en las pruebas de función hepática. El examen histológico reveló una reducción significativa de las áreas lesionadas. HGF también conservó la estructura de las uniones estrechas hepatocelulares. Estos efectos anticolestásicos se asociaron con la inducción de transportadores ABC de flujo basolateral, lo que facilita la extrusión de compuestos biliares tóxicos y su posterior depuración alternativa a través de la orina. El epitelio biliar parece haberse preservado también, como lo sugiere una normalización de los niveles séricos de GGT, la expresión de CFTR y la estructura del cilio primario de los colangiocitos. Finalmente, reportamos que HGF también protege a los riñones y su función del deterioro producido por la colestasis inducida por ANIT (nefropatía colémica). En conclusión, los resultados muestran claramente por primera vez que HGF protege el hígado y los riñones de una lesión colestásica.

**Abstract**

Cholestasis is a clinical syndrome common to a large number of hepatopathies, in which either bile production or its transit through the biliary tract is impaired due to functional or obstructive causes, respectively; the consequent intracellular retention of toxic biliary constituents generates parenchyma damage, largely via oxidative stress-mediated mechanisms. Hepatocyte growth factor (HGF) and its receptor c-Met represent the first defense line against hepatotoxic factors, by inducing Nrf2 activation which, in turn, leads to an antioxidant and repair response. In this study, we evaluated the capability of HGF to counteract the damage caused by the model cholestatic agent, α-naphthyl isothiocyanate (ANIT). HGF had clear anti-cholestatic effects, as apparent from the improvement in both bile flow and liver function test. Histology examination revealed a significant reduction of injured areas. HGF also preserved hepatocellular tight-junctional structures. These anticholestatic effects were associated with the induction of basolateral efflux ABC transporters, which facilitates extrusion of toxic biliary compounds and its further alternative depuration via urine. The biliary epithelium seems to have been also preserved, as suggested by a normalization in serum GGT levels, CFTR expression, and cholangyocyte primary cilium structure. Finally, we are reporting that HGF also protects the kidneys and their function from the impairment provoked by ANIT-induced cholestasis (cholemic nephropathy). In conclusion, the results clearly show for the first time that HGF protects the liver and kidneys from a cholestatic injury.

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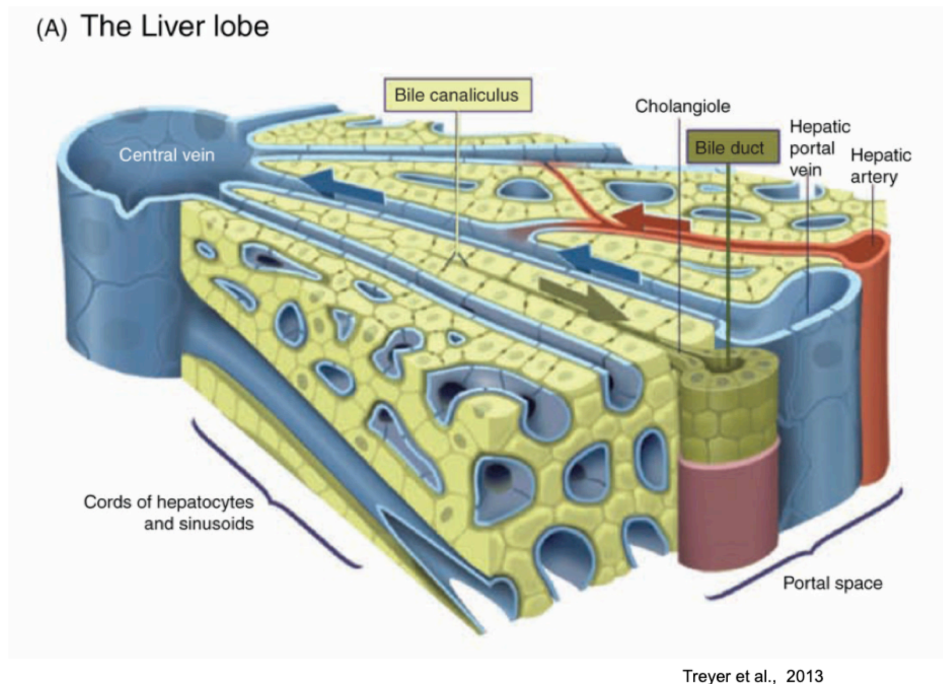
## 1. Introducción

### *1.1 Generalidades y morfología hepática*

El hígado es el segundo órgano más grande después de la piel, con un peso promedio de 1.5 kg en el adulto. Por el fluye un promedio de un litro y medio de sangre cada minuto, de ahí su color característico. El hígado realiza más de 500 funciones que se requieren para mantener la homeostasis del organismo, entre las que se encuentran la transformación de los alimentos en energía, la síntesis de factores de coagulación, proteínas, ácidos biliares y hormonas, la regulación del metabolismo de lípidos, el almacenamiento de algunas vitaminas y del glucógeno, principal fuente de energía la cual se reparte a todo el cuerpo en forma de glucosa. Adicionalmente, el hígado se encarga de la eliminación de sustancias tóxicas que ingerimos, respiramos o nos untamos en la piel (Kuntz 2006).

La estructura básica del hígado es el lobulillo hepático (Figura 1), donde la sangre circula desde las vénulas portales hasta la periféria, atravesando los sinusoides hepáticos hasta llegar a la vena central. Las vénulas portales, los conductos biliares terminales y las arteriolas hepáticas se disponen formando una triada o espacio portal (Isselbacher K. 1994). Los sinusoides hepáticos llevan la sangre mezclada de las ramas terminales de la vena porta hasta la vena central, y los hepatocitos contiguos a ellos producen bilis, la cual fluye en contraflujo hasta el ducto biliar del espacio portal. El parénquima hepático está conformado por un 78% de hepatocitos, 2.1% de macrófagos hepáticos (células de Kupffer), 2.8% de células endoteliales y 1.4% de células estelares (Kuntz 2006).




Treyer et al., 2013

***Figura 1. Lobulillo hepático y sentido del flujo sanguíneo y biliar.*** *Representación gráfica del lobulillo hepático con la triada o espacio portal formado por vena porta, artertia hepática y ducto biliar, del cual las flechas azul y rojo muestran el sentido del flujo sangunero que va desde el espacio potal a la vena central y en sentido contrario como muestra la flecha verde corre el flujo de la bilis.*

Los hepatocitos pueden realizar la mayoria de las funciones hepáticas (Arias I. AH 2009). Estas células, por formar parte de un epitelio secretor, se encuentran polarizadas, con dominios luminales y basales contiguos al lumen canalicular y la membrana basal, respectivamente, separados por las uniones estrechas, que unen los dominios laterales contiguos y restringen el pasaje de sustancias de plasma a bilis, manteniendo la hermeticidad del canalículo biliar (Treyer et al. 2013) (Figura 2).

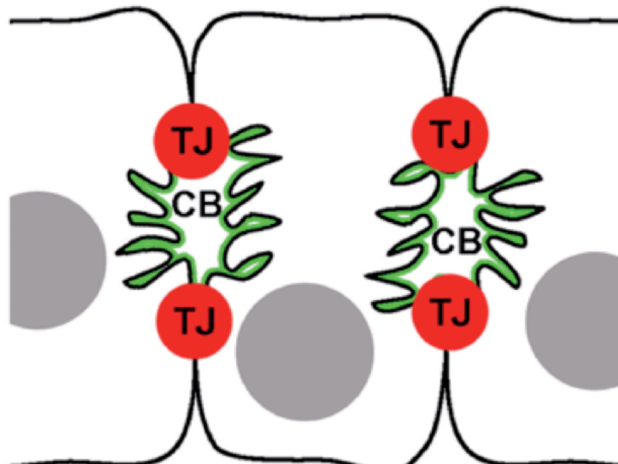



***Figura 2. Representación del canalículo biliar, el cual se encuentra sellado por uniones estrechas.*** *TJ, uniones estrechas ("tight junctions"); CB, canalículo biliar.*

### *1.2 El hepatocito y la formación de la bilis*

El hepatocito expresa sistemas de transporte diferenciales en sus distintos dominios de membrana plasmática, esto es la membrana apical (canalicular) y la basolateral (sinuoidal). Estos transportadores desempeñan un papel clave en la transferencia de solutos y agua desde el sinusoide y la sangre, lo que contribuye a la formación de la bilis y la excreción biliar de muchos xenobióticos (Roma et al. 2008).

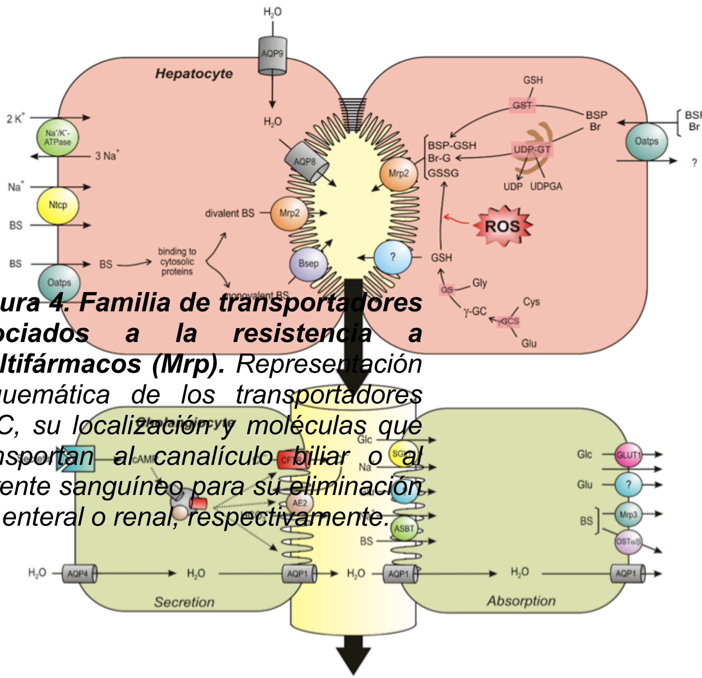
La bilis es un líquido amarillo-verdoso formado por agua, sales biliares, fosfolípidos, glutatión, bilirrubina, colesterol libre, iones de sodio, potasio, calcio y cloro, albúmina, toxinas endo y xenobióticas, además de algunas enzimas de membrana, como la fosfatasa alcalina (ALP). El pH biliar es de 6.2-8.5 (Kuntz 2006).

La formación de la bilis es un proceso osmótico impulsado por la excreción a un

espacio hermético, el canalículo biliar, de ciertos componentes biliares, tales como las sales biliares y el glutatión, con el consiguiente influjo de agua mediado principalmente por acuaporinas (Esteller 2008) (Figura 3). Las sales biliares son captadas por el hepatocito luego de haber sufrido recirculación enterohepática o se sintetizan a partir del colesterol en el hepatocito, siendo la enzima CYP7A1 el paso limitante del proceso. Las sales biliares provenientes de ambos procesos se excretan a la bilis por la bomba de exportación de sales biliares (BSEP o Abcb11). Este proceso es el punto de partida de la recirculación enterohepática de sales biliares, un ciclo por el cual las mismas son excretadas a bilis, volcadas al duodeno y reabsobidas en el intestino, siendo retornadas al hepatocito por sangre mesentérica para su recaptación (Hofmann 1984). Similarmente, otras sustancias son excretadas a través de la membrana canalicular del hepatocito por otros transportadores ABC ("ATP binding cassette") (Roma, Crocenzi et al. 2008), que son el paso limitante del transporte global de sustancias desde el plasma a la bilis, y por ende el principal factor determinante en la capacidad general del hígado para secretar bilis y para la desintoxicación de endo y xenobióticos (Roma et al. 2008).

Las sales biliares son esenciales para la emulsificación de la grasa dietaria y vitaminas lipofílicas, un proceso necesario para su digestión y absorción. Contribuyen también a balancear, junto con los fosfolípidos, el equilibrio para mantener disuelto el colesterol libre, y con ello, evitar la colelitiasis. Recientemente, se les ha atribuido relación con procesos de proliferación celular epitelial, expresión de genes y metabolismo de lípidos y glucosa por activación de receptores nucleares como FXR (Farnesoid X receptor),

PXR (Pregnane X receptor), VDR (Vitamin D receptor), receptores de membrana como GPBAR-1 (G-protein coupled bile acid receptor 1) o TGR5 y proteínas cinasas como JNK y ERK (Di Ciaula et al. 2017).




Roma et al., 2008

***Figura 3. Formación de la bilis primaria hepatocelular y su modificación ductular.*** *Se muestra esquemáticamente el transporte por el hepatocito desde el plasma al canalículo biliar de los componentes biliares que contribuyen a la formación de la bilis (sales bilires, glutatión) a través de los transportadores canaliculares, y el transporte osmótico de agua facilitado por aquaporinas. Una vez en el canalículo, la bilis viaja a través de los ductos biliares formados por colangiocitos, adicionando $HCO_3^-$ y agua, al ser estimulados por secretina en el período posprandial. En los períodos interprandiales, en cambio, predomina la reabsorción de diferentes solutos desde el lumen biliar (glucosa, sales biliares, $Na^+$, etc.), conformando un flujo ductular reabsortivo neto.*

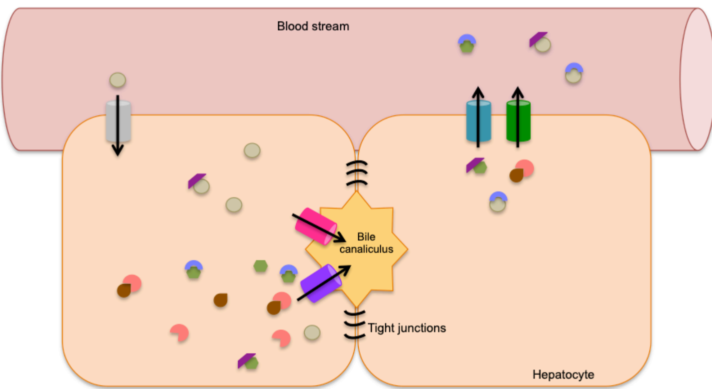



***Figura 4. Familia de transportadores asociados a la resistencia a multifármacos (Mrp).*** *Representación esquemática de los transportadores ABC, su localización y moléculas que transportan al canalículo biliar o al torrente sanguíneo para su eliminación vía enteral o renal, respectivamente.*

### *1.3 Sistemas de transporte ABC expresados por el hepatocito*

La familia de proteínas asociados a la resistencia a multifármacos (Mrp) representa el principal sistema transportador de compuestos a la bilis en los hepatocitos. La proteína Mrp2 es el principal transportador de glutatión, así como de los glucurónidos

de bilirrubina y una amplia gama de glucurónidos y glutatión conjugados de endo y xenobióticos. Además, otros miembros de esta familia de transporte, como Mrp3 y Mrp4, se ubican en la membrana basolateral, y median la extrusión de numerosas sales biliares y varios conjugados de glutatión, ácido glucurónico y sulfato hacia el torrente sanguíneo (Figura 4) (Roma et al. 2008).

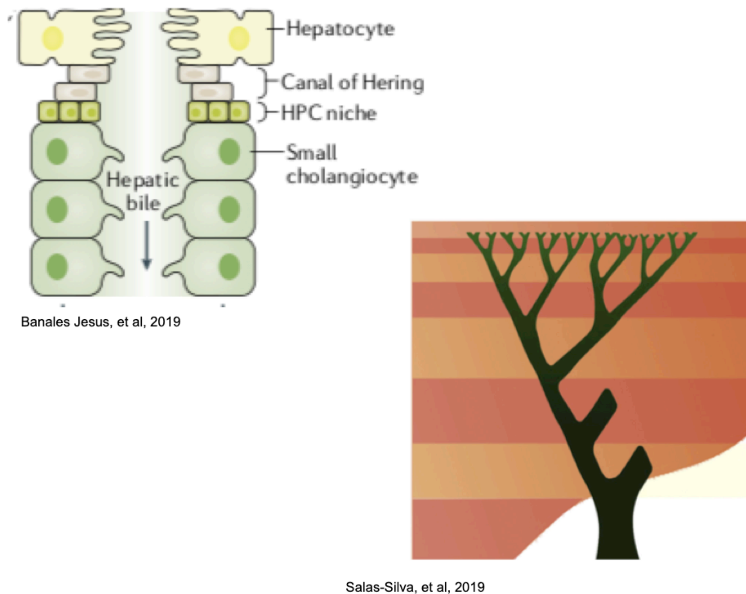



***Figura 5. Los colangiocitos y el árbol biliar a través del tejido hepático.*** *La bilis producida por el hepatocito se produce en el canalículo biliar, y a continuación encontramos el canal de Hearing y el nicho de células hepáticas progenitoras (HPC) que sirven de puente entre el hepatocito y los colangiocitos. Estas últimas células forman los conductillos biliares, ductos, etc. hasta el ducto biliar común, conformando una serie de conductos que comúnmente conocemos como "árbol biliar".*

La secreción primaria canalicular sufre modificaciones secundarias durante su paso por el árbol biliar, una compleja red tridimensional de conductos tubulares de varios tamaños y propiedades, cuyas paredes están formadas por colangiocitos (Figura 5). Estas células modifican la bilis al mediar el transporte coordinado de iones, solutos y agua a través de las caras apical y basolateral del

colangiocito,  participando en este proceso el cilio primario, una proyeccción de su membrana que le sirve como mecano-, quimio- y osmo-sensor de la calidad de la bilis (Figura 5) (Tabibian et al. 2013).

Por lo tanto, la formación, transporte y modificación de la bilis requiere no solo de integridad funcional, sino también de integridad estructural de los hepatocitos y colangiocitos (Tabibian, Masyuk et al. 2013).

### *1.4 Daño hepático y estrés oxidante*

Entre los principales agentes que afectan al hígado, se encuentran sustancias toxicas capaces de generar desbalances redox conducentes a estrés oxidante, tales como el alcohol, fármacos, drogas, el exceso de grasas y las infecciones virales, como la hepatitis B y C.

El estrés oxidante en el hígado también se exacerba en hepatopatías de tipo colestásico. En la colestasis, el flujo de bilis que llega al duodeno se encuentra disminuido, ya sea por impedimentos mecánicos que obstruyen el tránsito de la bilis (colestasis obstructiva) como por alteraciones funcionales en la capacidad del hepatocito para generar bilis (colestasis hepatocelular ó funcional). Las sales biliares y la bilirrubina retenidas por la falla secretora producen un exceso de radicales libres de origen mitocondrial por desacople de fosforilación oxidativa mitocondrial y formación de poros de transición de permeabilidad mitocondrial (Perez et al. 2009). Esto puede conducir a necrosis o apoptosis de hepatocitos y colangiocitos, dependiendo de la gravedad de la colestasis (Hofmann 1999); la necrosis sería el principal mecanismo de la muerte celular en la colestasis severa, mientras que la

apoptosis sería el predominante en condiciones más benignas (Benz et al. 1998). El estrés oxidante asociado a colestasis también inicia una respuesta inflamatoria, resultando en la acumulación de neutrófilos en el hígado, los cuales exacerban el estrés oxidante a través de la producción y liberación de radicales libres (Copple et al. 2010).

El estrés oxidante altera, a su vez, los mecanismos responsables de la producción de bilis, induciendo desorganización del citoesqueleto de actina, lo cual conduce al deterioro de las uniones estrechas y la internalización endocítica de transportadores canaliculares relevantes para la formación de la bilis (Roma, Crocenzi et al. 2008, Roma and Sanchez Pozzi 2008). Se establece entonces un círculo vicioso por el cual la falla secretora primaria ocasiona un desafío oxidante, que produce a su vez una alteración secretora secundaria de los hepatocitos que exacerba la alteración primaria secretora, perpetuando el daño colestásico (Roma and Sanchez Pozzi 2008). Para limitar la acumulación intracelular de sales biliares, en los hepatocitos colestáticos se establece una respuesta adaptativa conducente a incrementar su exportación basolateral de compuestos retenidos potencialmente tóxicos, como sales biliares y bilirrubina conjugada (Fickert et al. 2013) induciendo la expresión de transportadores basolaterales, tales como Mrp3, Mrp4 y OST$\alpha$/OSTß (Roma and Sanchez Pozzi 2008), haciendo posible así su depuración alternativa por vía renal.

Lo anterior a costa de aumentar la carga de sales biliares y bilirrubina en el sistema tubular renal, produciendo daño oxidante inducido por ambos compuestos, endotoxemia causada por la translocación de estos desde el intestino, resultando en

la falta enteral de sales biliares. Estas alteraciones renales en la colestasis se conocen como nefropatía colémica, que también incluye a la lesión renal aguda y las alteraciones morfológicas típicas de la histología renal en pacientes con ictericia que consisten en la formación de cilindros intratubulares (cast) y la lesión de las células epiteliales tubulares dirigidas hacia los segmentos distales de la nefrona. La nefropatía colémica se ha descrito en una variedad de escenarios clínicos tales como la colestasis obstructiva, lesiones hepáticas inducidas por fármacos, hepatitis fulminante, esteatohepatitis alcohólica y cirrosis hepática (Fickert, Krones et al. 2013, Krones et al. 2018).

La permeabilidad biliar está estrechamente relacionada con la regulación funcional y la integridad de las uniones estrechas (zonula occludens). Estas estructuras sellan el lumen de los canalículos biliares entre hepatocitos adyacentes, delineando así el lumen canalicular y reteniendo selectivamente el contenido de la bilis dentro de ellos. La evidencia de estudios fisiológicos y morfológicos indica que las uniones estrechas tienen permeabilidad selectiva para ciertos solutos, lo que sugiere un papel regulador de las mismas en la formación de bilis. En línea con este punto de vista, se ha demostrado que la permeabilidad a lo largo de esta vía es modulada por una variedad de reguladores fisiológicos de la secreción biliar, tales como los ácidos biliares y varias hormonas (Roma et al. 1995).

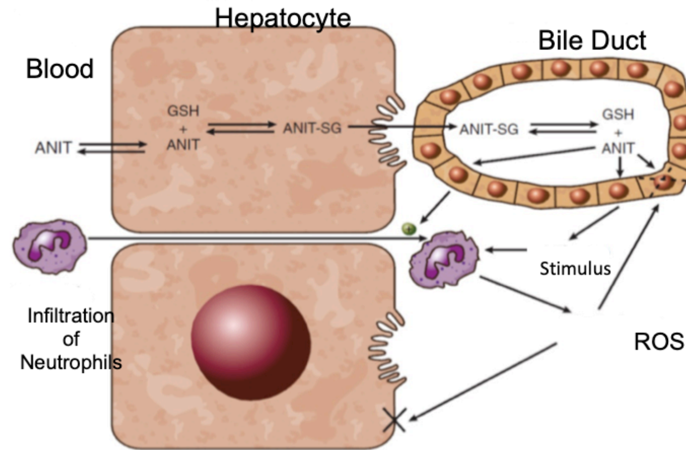
### *1.5 Colestasis inducida por α-naftil isotiocianato (ANIT)*

El ANIT es un compuesto químico hepatotóxico que se ha sido utilizado principalmente como modelo para estudiar la patogénesis de la colestasis, particularmente aquella

inducida por fármacos. La colestasis inducida por ANIT también ha sido usada como modelo para estudiar los efectos de la colestasis en el metabolismo (Dahm et al. 2010). Se sabe que la colestasis inducida por este fármaco se desarrolla en dos fases. En la fase temprana (< 48h), la colestasis resulta de deficiencias funcionales (alteración de la función de transportadores canaliculares, aumento de la permeabilidad paracelular). En tiempos posteriores (desde las 48-72 h después del tratamiento), hay obstrucción biliar por descamación de las células epiteliales del ducto biliar, por el ataque del ANIT a los colangiocitos, produciendo necrosis, tardía hiperproliferación ductular e infiltración inflamatoria asociada a neutrófilos como consecuencia de la obstrucción. El ANIT es destoxificado en el hepatocito a través de su conjugación con el glutatión (GSH). El complejo ANIT-GSH es transportado por Mrp2 a la bilis, donde ANIT se disocia del GSH por la alcalinidad biliar (Figura 6). La liberación de ANIT daña progresivamente a las células epiteliales de los conductos biliares, siendo este daño exacerbado por su reciclaje a través de recirculación enterohepática, consistente en ciclos repetidos de captación hepática, conjugación con GSH, excreción biliar, desconjugación luminal, reabsorción intestinal de la forma no conjugada, y subsiguiente recaptación (Tanaka et al. 2009, Yang et al. 2013). Por las características de su mecanismo colestásico inflamatorio y obstructivo del árbol biliar, la colestasis inducida por ANIT en murinos ha sido propuesta como un modelo que mimetiza la colangitis esclerosante en humanos (Fickert et al. 2014).

Se ha demostrado que ANIT incrementa la permeabilidad hepatocelular y/o permeabilidad y morfología de las uniones estrechas de las células epiteliales biliares

antes del inicio de la colestasis, lo cual coincide con la disminución del flujo biliar (Jaeschke et al. 1987).




### *1.6 Mecanismos de defensa hepática: El factor de crecimiento de hepatocitos (HGF)*

***Figura 6. Mecanismo de daño por ANIT.*** *Una vez dentro del hepatocito, el ANIT es conjugado con GSH (ANIT-SG) para su eliminación; esta se lleva a cabo a través del transportador canalicular Mrp2. Una vez en el lumen canalicular, el conjugado ANIT-SG se disocia, y el ANIT libre resultante ejerce efecto tóxico sobre los colangiocitos y muerte celular. La presencia de ANIT también induce el reclutamiento de células proinflamatoria como los neutrófilos, agravando así el daño inducido por ANIT.*

HGF y su receptor de membrana, el factor de transición mesénquima-epitelio celular (c-Met), representan la primera línea de defensa contra los factores hepatotóxicos. En los hepatocitos, el HGF se identificó originalmente como un mitógeno potente. Es una proteína dimérica de 84 kDa, que se compone de una subunidad α de 69 kDa y una subunidad β de 34 kDa, unida por un puente disulfuro y se produce en células del estroma, como las células estelares (Nakamura et al. 1989). Se sintetiza como pro-HGF, de 728 aminoácidos (Nakamura, Nishizawa et al. 1989, Nakamura et al. 2000), y luego madura en HGF por medio de una escisión proteolítica. En las etapas fetales,

la neutralización de HGF o el silenciamiento de c-Met conducen a la hipoplasia de varios órganos, lo que indica que la señalización mediada por este factor de crecimiento es esencial para el desarrollo de los órganos. Se requiere HGF para reparar el daño pulmonar, hepático (Fausto 2000) y renal (Matsumoto et al. 2001), entre otros, además de tener efectos protectores en órganos epiteliales y no epiteliales, como el corazón y el cerebro (Nakamura, Mizuno et al. 2000).

El HGF se considera actualmente un factor de crecimiento multitarea implicado en muchos roles celulares en el hígado, como la motilidad, la morfogénesis y la supervivencia. El HGF funciona como un modulador del sistema del glutatión (GSH), que es un determinante fundamental del estado redox celular (Gomez-Quiroz et al. 2008, Deheuninck et al. 2009). Este hecho evidencia que la señalización de c-Met es un factor crítico en la protección contra la sobregeneración de especies reactivas de oxígeno (ROS) (Gomez-Quiroz, Factor et al. 2008). Por ello, se ha caracterizado el papel de HGF/c-Met en la regulación del estado redox celular y el estrés oxidante en el hígado (Enriquez-Cortina et al. 2013). HGF, interaccionando con su receptor de membrana c-Met con actividad de tirosina cinasa (Gomez-Quiroz, Factor et al. 2008, Enriquez-Cortina, Almonte-Becerril et al. 2013), regula la activación de factores de transcripción claves que impulsan la expresión de genes antioxidantes y de supervivencia, como el factor nuclear κ B (NF-κB) o la proteína cinasa C delta (PKCδ), dependiente del factor de transcripción Nrf2, el cual conduce a una respuesta de

supervivencia celular bajo agresiones de estrés oxidante (Figura 7) (Clavijo-Cornejo et al. 2013).

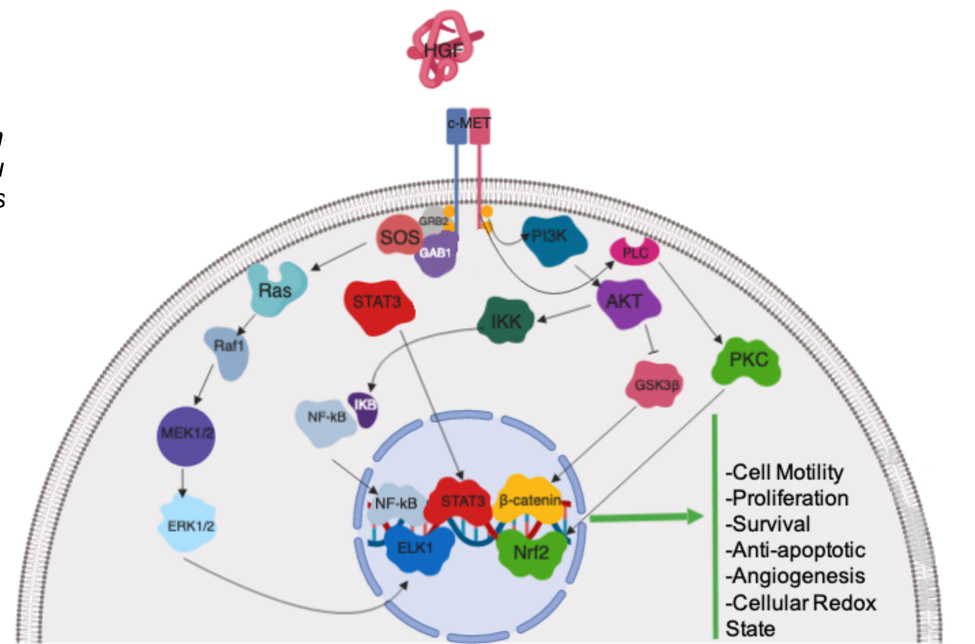


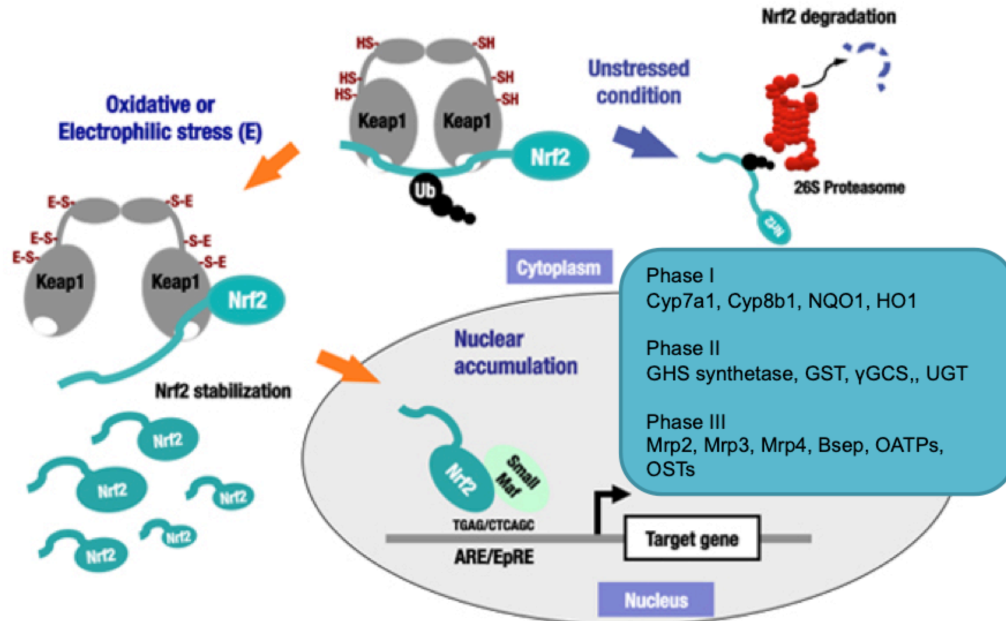

***Figura 7.*** *...on su receptor m... ...eínas que condu... ...entes respuestas...*

### *1.7* Defensas antioxidantes activadas por Nrf2

El factor de transcripción Nrf2 (factor 2 relacionado con el factor nuclear eritroide 2 p45) regula en el hígado la expresión de una amplia gama de genes antioxidantes y

citoprotectores, como los que codifican a las enzimas de fase II NADPH deshidrogenasa quinona 1 (NQO1), ɣ-glutamil cisteína sintetasa (ɣ-GCS), glutatión *S*-transferasa (GST), hemooxigenasa 1 (HO1) y uridina difosfato-glucurosiltransferasa (UGT), así como también transportadores hepatocelulares, como Mrp2, Mrp3, Mrp4, Bsep, OATP´s y OSTα, y a enzimas de fase I, tales como Cyp7a1 y Cyp8b1 (Weerachayaphorn et al. 2012, Clavijo-Cornejo, Enriquez-Cortina et al. 2013).

En condiciones normales, Nrf2 reside en el citoplasma, formando un complejo inactivo con su inhibidor Keap1, una proteína adaptadora para la ligasa Cullin3, enzima que media la degradación proteolítica de Nrf2. Después de un estímulo mediado por ROS, se oxidan residuos de cisteína específicos en Keap1, lo cual libera a Nrf2, quien migra al núcleo para iniciar la transcripción de enzimas antioxidantes de fase II, involucradas en la síntesis de glutatión, y de fase III, involucradas en la desintoxicación, como los transportadores ABC (Figura 8) (Clavijo-Cornejo, Enriquez-Cortina et al. 2013).




Modificada de Masayuki Yamamoto

***Figura 8. Mecanismo activación de Nrf2.*** *En condiciones normales, Nrf2 reside en el citoplasma anclado a su inhibidor Keap1. Si Nrf2 no es requerido, este es degradado vía proteosomas. Sin embargo, en condiciones de estrés oxidante, las ROS oxidan a Keap1, y así Nrf2 puede ser liberado para su translocación al núcleo e inducir la transcripción de sus genes blanco, para conducir así a una respuesta antioxidante.*

## 2. Justificación

Las estadísticas en salud, de conformidad con lo informado por la Secretaría de Salud en el Programa Sectorial de Salud 2013-2018 publicado en el Diario Oficial de la Federación el Jueves 12 de diciembre de 2013 (ver el sitio web de la secretaría: http://www.dgis.salud.gob.mx/contenidos/sinais/e_mortalidadgeneral.html), muestran que las enfermedades crónicas del hígado ocupan el cuarto lugar de las principales

causas de mortalidad general a nivel nacional, mientras que en la población con edad productiva (15-64 años) ocupan el segundo lugar, por lo que es clara la importancia que tienen las enfermedades hepáticas como problema de Salud Pública en nuestro país.

Actualmente, no existe un tratamiento efectivo para las enfermedades colestáticas debido al escaso conocimiento de su patogénesis. Esta es la razón por la cual las enfermedades colestáticas son la principal causa de enfermedad asociada al hígado, lo que lleva a cirrosis e insuficiencia hepática progresiva que requiere un trasplante de hígado para que el paciente sobreviva (Vij et al. 2015). Aunque se han informado algunos enfoques limitados con respecto al efecto protector del HGF en la colestasis inducida por ANIT en la rata (Roos et al. 1992), el mecanismo de acción sigue sin estar claro. Por esta razón, el objetivo de este trabajo fue caracterizar el efecto anti-colestásico del HGF en un modelo murino de colestasis química inducida por ANIT.

## 3. Pregunta de investigación

¿Ejercerá el HGF un efecto protector a nivel hepático y renal del daño colestásico inducido químicamente por el ANIT?

## 4. Hipótesis

El HGF ejercerá un efecto protector en la colestasis inducida por el ANIT en un modelo en animales de experimentación, controlando el estrés oxidante y derivando las sales biliares hacia la vía sanguínea y renal, a través de regular diferencialmente transportadores hepatobiliares, preservando así la función hepática y renal.

## 5. Objetivos

### *5.1 Objetivo general*

Caracterizar el papel protector del HGF a nivel hepático y renal en un modelo de colestasis química inducida por el ANIT.

### *5.2 Objetivos particulares*

1. Determinar el daño colestásico hepático en ratones sometidos a un tratamiento con ANIT, y el efecto protector del HGF en dicho modelo.
2. Determinar el estrés oxidante inducido por el ANIT y el efecto antioxidante ejercido por el HGF en dicho modelo.
3. Caracterizar la expresión diferencial de transportadores de sales biliares y bilirrubina en animales tratados con ANIT y sus modificaciones con el co-tratamiento con HGF.
4. Caracterizar los cambios que produce el ANIT en el flujo biliar y en la excreción biliar de los compuestos que los generan, y los efectos del co-tratamiento con HGF sobre los mismos.
5. Determinar los cambios estructurales a nivel del parénquima hepático y de la vía biliar en ratones sometidos al ANIT y en el co-tratamiento con HGF.
6. Determinar los cambios estructurales y funcionales a nivel renal en animales tratados con ANIT y los efectos de co-tratamiento con HGF.

## 6. Diseño Experimental

### *6.1 Modelos en animales*

Se usaron ratones macho de la cepa CD-1, de 10-12 semanas de edad, los cuales fueron obtenidos del bioterio de la UAM Iztapalapa. Los animales se mantuvieron en

condiciones estándar con ciclos de luz obscuridad de 12 h, en condiciones controladas de humedad y temperatura. Los procedimientos fueron aprobados por el Comité de Ética de la UAM-I y se siguieron los lineamientos para el uso y manejo de animales de laboratorio de los institutos Nacionales de Salud de Estados Unidos (NIH) y los de la Normal Oficial Mexicana (NOM,) **NOM**-062-ZOO-**1999.**

### *6.1.1 Modelo de daño agudo con ANIT*

40 ratones CD-1 se dividieron aleatoriamente en 4 grupos y se colocaron individualmente en jaulas metabólicas, con alimento estándar y agua *ad libitum*. Los ratones recibieron una dosis de ANIT 60 mg/kg, i.g. o vehículo durante 48 h. Después de 24 h del tratamiento con ANIT, se administró HGF 10 µg/kg, i.v. Se recolectó la orina de las últimas 12 h de los animales. Cuarenta y ocho horas después del tratamiento con ANIT, los animales fueron sacrificados bajo anestesia con isoflurano (2% de isoflurano y 2 l/min de oxígeno). Se obtuvieron sangre, bilis y tejido hepático y renal.

## *6.2 Materiales y Métodos*

### *6.2.1 Determinaciones bioquímicas en suero y orina*

Se obtuvieron muestras de sangre del plexo venoso orbitario bajo anestesia (2% de isoflurano y 2 l/min de oxígeno). Las enzimas séricas de daño hepático aspartato aminotransferasa (AST), alanina aminotransferasa (ALT), fosfatasa alcalina (ALP) y γ-glutamil transpeptidasa (GGT) se determinaron mediante el uso de un método automatizado (SpotChem EZ, ARKRAY, EE. UU.). La creatinina en suero y orina se determinó mediante QuantiChrom Creatinine Assay Kit (BioAssay Systems, USA).

### *6.2.2 Determinación de sales biliares y bilirrubina*

El contenido de sales biliares en suero, tejido y orina se analizó mediante Total Bile Acid Assay (DIAZYME Laboratories, CA, USA), y la bilirrubina y sus glucuronoconjugados a través del Jendrassik-Grof FS kit (DiaSys Inc, Canada), siguiendo las instrucciones del fabricante.

### *6.2.3 Análisis histológico*

La histología se realizó por tinción de Hematoxilina-Eosina (H-E). Para ello, los portaobjetos conteniendo los cortes de hígado incluidos en parafina se introdujeron 10 min en la estufa a 60° C. Las secciones de tejido hepático de 8μM fueron desparafinadas durante 10 min con xilol, y gradualmente se hidrataron con alcoholes del 100% al 70% durante 5 min, seguido de agua durante otros 5 min. Después, se colocaron en hematoxilina de Harris durante 5 min. Consecutivamente, se hizo un lavado de estos en agua durante 5 min, se sumergieron en HCl-EtOH al 96% hasta la aparición de un color rojizo, y después se sumergieron en agua amoniacal (200 ml de agua destilada con 3-4 gotas de amoníaco), hasta que apareció un color azul. Luego, se volvió a lavar con agua corriente durante 2 min y con agua destilada otros 2 min y, enseguida, se colocaron los portaobjetos en eosina alcohólica durante 2 min. Posteriormente, se deshidrataron durante 1 min en solución de etanol de diferente concentración pasando del etanol de 70% al absoluto, hasta llegar al xilol. Al finalizar, se montaron con Dako Cytomation.

### *6.2.4 Estudios de flujo biliar*

La bilis se recolectó bajo anestesia con isoflurano. El conducto biliar común se canuló con un tubo de polietileno (PE10) (Figura 15C). Se colectó la bilis durante 30 minutos, y se determinó el flujo biliar gravimétricamente, asumiendo una densidad de 1 g/ml, como se informó anteriormente (Manautou et al. 2005).

#### *6.2.5 Eliminación de sales biliares en bilis*

Las sales biliares totales se determinaron mediante el procedimiento de la 3α-hidroxiesteroide deshidrogenasa (Basiglio et al. 2014).

#### *6.2.6 Determinación de especies reactivas del oxígeno (ROS) in situ*

Después de la eutanasia de los ratones, se obtuvo rápidamente tejido hepático y renal fresco y se seccionó, se congeló en nitrógeno líquido y se embebió en reactivo de temperatura de corte óptimo (OCT, Sakura Finetec, Torrance, CA, EE. UU.). Inmediatamente, se obtuvieron secciones congeladas de 8 μm en un criostato (Leica CM-3050S) a -20° C, y los portaobjetos se incubaron durante 15 minutos con dihidroetidio (DHE, 5 μM # D11347, Invitrogen, CA, EE. UU.), en la oscuridad y a temperatura ambiente, para la determinación de fluorescencia de etidio, que detecta radicales superóxido. Las muestras se observaron utilizando un microscopio confocal (Carl Zeiss 780 LSM-NLO), a longitudes de onda de excitación y emisión de 485 y 570 nm, respectivamente, como informamos previamente (Enriquez-Cortina et al. 2017).

#### *6.2.7 Oxidación lipídica*

La oxidación lipídica, un parámetro subrogado de la generación de ROS intercelular, se analizó mediante la producción de sustancias reactivas al ácido tiobarbitúrico

(TBARS), utilizando un método espectrofotométrico (Dominguez-Perez et al. 2018).

### *6.2.8 Ensayo de retardo de movilidad electroforética (EMSA)*

El ensayo se realizó con el estuche comercial Light Shift Chemiluminescent EMSA (Thermo Scientific #20148, E.U.A.), siguiendo las especificaciones del fabricante. El tejido se homogenizó, y se le agregó buffer de lisis. A continuación, se centrifugó a 1470 x g por 5 min a 4° C, se obtuvo el botón nuclear y se resuspendió en buffer de extracción y de nuevo se centrifugó a 15 500 x g por 10 min a 4° C. Se preparó un gel de poliacrilamida al 4-6% en TBE 0.5X, y se pre-corrió de 30 a 60 min a 100 V. Para las reacciones, se descongelaron los reactivos en hielo, y se prepararon de acuerdo con la tabla que contiene el kit (estos reactivos son específicos para las proteínas de interés, en este caso Nrf2), dejándose a temperatura ambiente durante 20 min. Después, se agregaron 5 µl de amortiguador 5X por cada 20 µl de reacción, homogenizando a la mezcla con una pipeta. Finalmente, se cargaron los pozos del gel con 20 µl de la muestra preparada, y se dejaron correr a 100 V hasta ¾ del gel. Mientras las muestras corrían en el gel, la membrana de nylon se remojó en TBE 0.5 X durante 10 min. Una vez que el gel corrió y se tuvo lista la membrana, se llevó a cabo la transferencia de la misma manera que un Western blot. Luego, se realizaron varios lavados a la membrana. Para detectar las proteínas que se marcaron, se utilizaron reactivos especiales del kit.

### *6.2.9 Estudios de integridad canalicular por inmunofluorescencia y análisis de imagen*

Para evaluar el estado estructural de las uniones estrechas paracelulares que sellan el canalículo biliar, se estudió la inmunolocalización de la proteína ZO-1 asociada a la unión estrecha por microscopía confocal (Zeiss Pascal LSM 5, Carl Zeiss, Alemania). Para este propósito, se extirpó un lóbulo hepático y se congeló inmediatamente en isopentano preenfriado en nitrógeno líquido, y se almacenó a -80° C. Las muestras de hígado se seccionaron y fijaron, y ZO-1 se marcó con anticuerpo policlonal (# 617300, Invitrogen, CA, EE. UU.). La inmunotinción se completó mediante el tratamiento de las preparaciones con anti-IgG de ratón conjugado con Cy3 (Jackson Immununo Research Laboratory, Inc. West Grove PA). El análisis de las imágenes se realizó utilizando el software Image J 1.34 m (NIH). El ancho canalicular promedio de cada grupo se evaluó como un primer enfoque para corregir las distorsiones en la intensidad de fluorescencia inducidas por cambios en el volumen canalicular, como resultado de los tratamientos. También se realizó una evaluación del porcentaje de canaliculos de 'doble riel' en imágenes confocales. Para evaluar la proporción de estructuras de doble riel canalicular, se cuantificó la presencia de rieles canaliculares simples y dobles, y luego, el número de estructuras de doble riel se relativizó al total de estructuras canaliculares (riel simple y doble). Alternativamente, las estructuras canaliculares se abordaron en el tejido hepático de ratones c-MetKO usando reactivo de rodamina-faloidina (# 235138, Abcam) en combinación con el anticuerpo IF ZO-1. El tejido del hígado de ratones c-MetKO fue generosamente donado por el profesor Gómez-Quiroz.

### *6.2.10 Cuantificación de proteína*

Se realizó la extracción de proteína usando un buffer de lisis con inhibidores de proteasas (PhosSTOP, Rocher, Complete, Rocher). Se realizó la cuantificación de la proteína mediante el estuche comercial de ácido bicinconínico (BCA, Pierce, Thermo Fisher Scientific), siguiendo el protocolo del fabricante.

| Anticuerpos | kDa | Marca | Dilución en WB |
|---|---|---|---|
| ZO1 | 195 | Invitrogen (617300) | 1:1000 |
| Bsep | 146 | Abcam (ab112494) | 1:1000 |
| Mrp2 | 174 | Abcam (ab3375) | 1:1000 |
| CFTR | 168 | Abcam (ab2784) | 1:1000 |
| Mrp3 | 190 | Abcam (ab110740) | 1:1000 |
| Mrp4 | 140-200 | Cell Signaling (12705) | 1:1000 |
| HSP72 | 72 | Enzo (C92F3A-5) | 1:1000 |
| Actin | 42 | Sigma Aldrich (a2060) | 1:10000 |

*Tabla. 1 Lista de anticuerpos usados en el presente trabajo.*

### *6.2.11 Western blot*

El western blot se realizó siguiendo el protocolo previamente reportado (Gerardo-

Ramirez et al. 2019). Se usó membrana de PVDF (Bio-Rad, USA) con los anticuerpos descritos en la Tabla 1. Las membranas fueron expuestas usando Super Signal West Pico Quimioluminiscente (Pierce Biotechnology, USA) y se revelaron usando un Gel Logic 1500 (Kodak, Rochester, NY, USA). Para el Western blot de orina conducente a determinar los niveles urinarios de HSP72, la orina se diluyó 1: 100 en solución salina al 0,9% y se cargaron 10 µl de cada dilución (Barrera-Chimal et al. 2011). Las bandas se cuantificaron por densitometría, usando el fotodocumentador Gel logic 1500 (Kodak, Rochester, NY, USA).

#### *6.2.12 Determinación de albuminuria*

Para determinar la cantidad de albúmina excretada a orina, se analizaron 5 µl de muestras de orina mediante electroforesis en gel de poliacrilamida al 4–20%. Para ello, el gel se incubó con azul brillante Coomassie R-250 (Bio-Rad # 161-0400) (Russo, Srivatsan et al. 2013). La concentración de albúmina en la orina en sí misma no es una buena medida de fuga de albúmina por vía renal, ya que su concentración se ve directamente afectada por el grado en que los riñones concentran la orina. Para compensar estas variaciones en la concentración urinaria de albúmina (o de HSP72) en muestras puntuales de orina, sus concentraciones fueron relativizadas a la concentración de creatinina, cuyas variaciones reflejan dichos cambios en el volumen urinario (Nisell et al. 2006).

#### *6.2.13 Estudio de la estructura del conducto biliar mediante microscopía electrónica de barrido (SEM).*

Las micrografías SEM (scanning electron microscope) se obtuvieron con un

microscopio electrónico de barrido (JEOL JSM-5900 LV). Todas las muestras se fijaron en glutaraldehído al 3% durante 48 h, se incubaron con tetraóxido de osmio durante 2 h y se deshidrataron en etanol al 30-100%. Después de secar usando el secador de punto crítico, las muestras se revistieron con oro. El SEM se hizo funcionar a 15 kV de aceleración de voltaje (Wisse et al. 2010).

### *6.2.14 Determinación de la expresión génica por RT-PCR en tiempo real*

Se realizó la cuantificación de los niveles de ARNm para genes seleccionados. El ARN total se obtuvo usando el reactivo de aislamiento de ARN TRIzol (# 15596018, Thermo Fisher Scientific, EE. UU.), siguiendo las instrucciones de fabricación. Después de la digestión con DNasa con un kit sin ADN (Ambion Inc, Thermo Fisher Scientific, EE. UU.), se transcribió inversamente 1 µg de ARN total en 20 µl de volumen de reacción con un SuperScript (# 170-8891 Invitrogen, CA, EE. UU.) kit de síntesis de primera cadena, de acuerdo con las instrucciones del fabricante. El análisis de PCR cuantitativo en tiempo real se realizó con un termociclador CFX96 Touch (Bio-Rad) en una placa de reacción de 96 pocillos. La mezcla de reacción de PCR de 10 µl contenía 5 µl de mezcla maestra 2X SYBR Green PCR (# 172-5016 Bio-Rad Laboratories, EE. UU.), 200 nM de cada cebador y 1 µl de plantilla de ADNc. Las reacciones se incubaron durante 10 minutos a 95 °C, seguidas por 40 ciclos de 30 segundos a 95 °C y 60 segundos a una temperatura específica del cebador. El análisis de fusión de los productos de PCR también se realizó para validar la amplificación del producto específico. El nivel de expresión de la proteína ribosómica del ratón S18 (rps18) se

utilizó como referencia interna. Los niveles relativos de expresión génica se calcularon con el método 2-ΔΔct. Las secuencias de primers utilizadas en el estudio se enumeran en la Tabla 2.

### *6.2.15 Análisis estadístico*

| Nombre de la proteína | Acronimo | Gen | Foward | Reverse |
|---|---|---|---|---|
| Bile salt efflux pump | Bsep | *Abcb11* | GGCTTGCTACAGATGC TTCC | GCCAAAAAGGGG AAGAAGAC |
| Multidrug resistance-associated protein 2 | Mrp2 | *Abcc2* | CTGAGTGCTTGGACCA GTGA | CCCACAGTCACCA TCCTCTT |
| Multidrug resistance-associated protein 3 | Mrp3 | *Abcc3* | CCCTGCTCCTGTCTTCT TTG | GCTGAGAGGATCT TGGAACG |
| Multidrug resistance-associated portein 4 | Mrp4 | *Abcc4* | CATACCATTGGTTCCGC TCT | GTCAGGAGCTGTT TGATGCA |
| Ribosomal protein s18 | s18 | *rps18* | TGTGGTGTTGAGGAAA GCAG | TCCCATCCTTCAC ATCCTTC |

*Tabla. 2. Lista de primers usados en el presente trabajo*

Los datos se presentan como media ± SEM para al menos 4 animales diferentes; cada experimento se llevó a cabo por triplicado. Para la comparación de medias de diferentes grupos, se utilizó un análisis de varianza (ANOVA), seguido de comparaciones múltiples mediante la prueba de Tukey. Se utilizó el software Graph Pad Prism versión 8 para MacOS. El nivel de significación considerado fue $p \leq 0.05$.

## 7. Resultados

**El ANIT induce daño hepatobiliar de una forma dependiente del tiempo.**

Con la finalidad de confirmar el daño hepato y colangiocelular inducido por el ANIT a una dosis de 60 mg/Kg, i.g, dosis reportada previamente (Kossor et al. 1993, Tanaka, Aleksunes et al. 2009), se precedió a caracterizar el daño del agente tóxico. Primero, se evaluaron los marcadores canónicos de daño y función hepática en un estudio curso-temporal. La Figura 9A muestra un incremento notable en la actividad sérica de AST, un buen indicador de daño por necrosis, desde las 12 h de tratamiento en adelante, efecto observado incluso hasta las 48 h. Por otro lado, los niveles de bilirrubina total (Figura 9B) y directa (Figura 9C) se elevaron más tardíamente, siendo esos incrementos significativos pasadas las 24 h de tratamiento, indicando un claro compromiso hepatocelular con impacto funcional.

El daño hepatocelular ocasionado por la retención intracelular de sales biliares se evaluó utilizando como marcador la enzima ALP. Esta enzima se encuentra unida a la membrana plasmática que es inducida y liberada en el torrente sanguíneo por el efecto tensoactivo de las sales biliares endógenas detergentes retenidas por el proceso colestásico (Kaplan et al. 1970), y representa una indicación temprana de daño en la membrana plasmática que conduce a la lisis hepatocelular asociada a este fenómeno (Billington et al. 1980). Hemos observado un incremento significativo desde las 36 h, sugiriendo que el daño hepatocelular ocasionado por ANIT *per se* es previo al daño provocado por las sales biliares retenidas como consecuencia secundaria del daño funcional sobre la excreción de sales biliares (Figura 10A). Esto coincide con el incremento del contenido de las sales biliares en suero que, como se muestra en la Figura 10B, también incrementan desde las 36 h, disminuyendo ligeramente a las 48 h. El incremento de las sales biliares a las 36 h es consecuencia de la falla secretora

biliar de las mismas y su redireccionamiento a plasma como consecuencia de la inducción adaptativa de bombas exportadoras basolaterales de sales biliares (por ej., Mrp3, Mrp4) (Zollner et al. 2006). En cambio, la disminución posterior a las 48 h podría ser resultado de la inducción adaptativa de sistemas de depuración renal de sales biliares, como ha sido reportado (Brandoni et al. 2012).

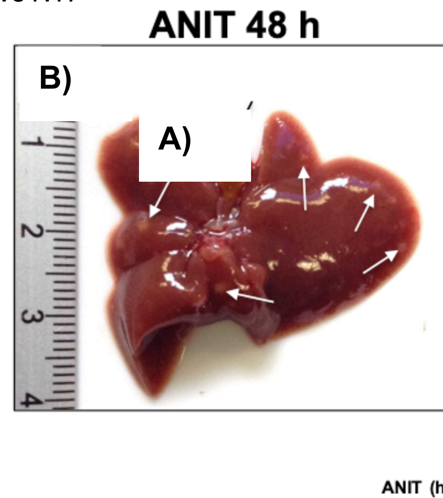
Estos resultados muestran que es a las 48 h cuando se observa un daño bien establecido. La inspección del órgano (Figura 11B) reveló un cambio en la coloración del hígado hacia un tono más pálido y la presencia de lesiones que sugieren zonas necrosadas (flechas blancas). La vesícula biliar mostró una ligera hipertrofia y coloración obscura (flecha negra). El hígado reveló un incremento en su tamaño (hepatomegalia), determinado por el cociente del peso del órgano con respecto al peso corporal (Figura 11C).

Con la finalidad de confirmar el daño necrótico, se realizaron estudios histológicos rutinarios con hematoxilina y eosina (H&E). La observación microscópica muestra áreas prominentes con claros signos de necrosis hepatocelular (asteriscos, Figura 12B), comparado con el tejido de animales no tratados que muestran una histología normal (Figura 12A). Las microfotografías muestran claramente que el ANIT induce daño hepatocelular necrótico (asteriscos), así como infiltrado inflamatorio (flechas).

Con base en los datos anteriores, y dado que el daño colestásico se ve plenamente establecido a las 48 h de tratamiento, decidimos continuar los estudios a 48 h de tratamiento con ANIT para estudiar el efecto anticolestásico del HGF.

A) B) C)

AST (U/L)




B)

#

Liver/Body weigth

0.09
0.08
0.07
0.06
0.05
0.04

NT 12 24 36 48

ANIT (h)

***Figura 9. ANIT induce daño hepático.*** *A) AST, B) bilirrubina total y C) bilirrubina directa. #p<0.01 vs NT.*

B)

ANIT (h) ANIT (h)

***Figura 11. Cambios macroscópicos inducidos por ANIT en el hígado.*** *#p<0.01 vs NT.*

***Figura 10. Efecto del ANIT sobre parámetros bioquímicos de colestasis****. A) Niveles séricos de ALP. B) Contenido de sales biliares en suero. #p<0.01 vs NT.*

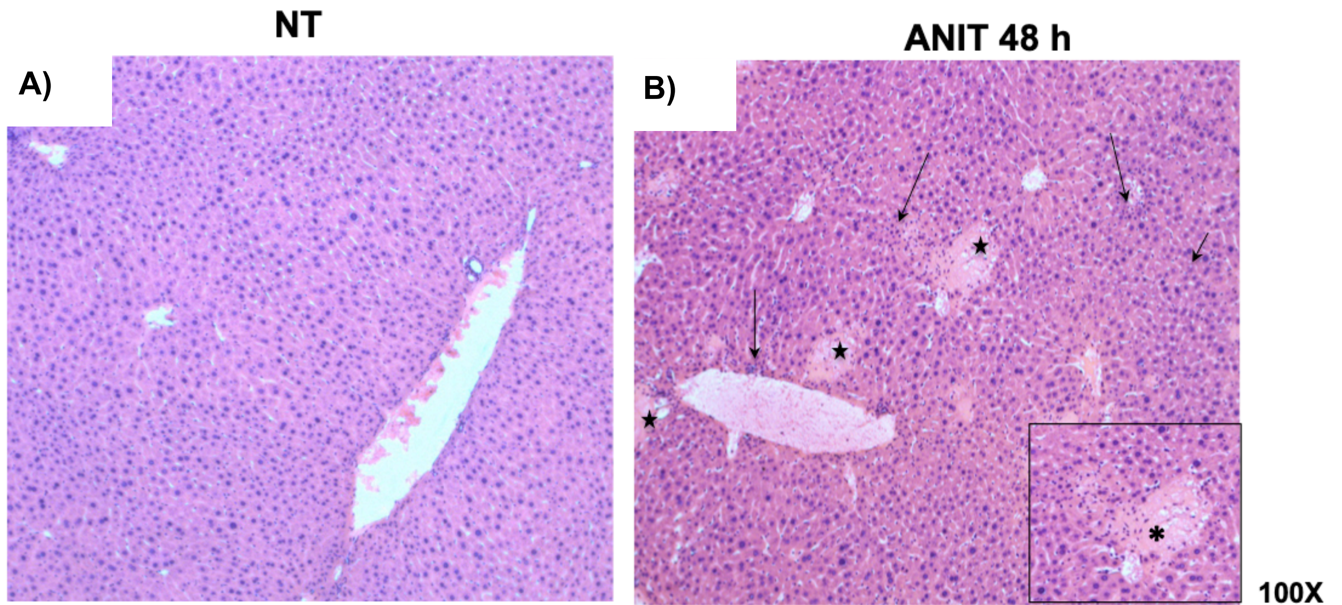



***Figura 12. El tratamiento con ANIT induce áreas necróticas e infiltrado inflamatorio.*** *Apariencia histológica por tinciones de H-E. Las estrellas negras indican las áreas con necrosis y las fechas muestran el infiltrado inflamatorio.*

## El HGF despliega propiedades anticolestásicas

Previamente, nuestro grupo de investigación ha reportado numerosas propiedades hepatoprotectoras del HGF (Gomez-Quiroz, Factor et al. 2008, Valdes-Arzate et al. 2009, Marquardt et al. 2012, Clavijo-Cornejo, Enriquez-Cortina et al. 2013, Enriquez-Cortina, Almonte-Becerril et al. 2013). Para demostrar que el HGF induce efectos protectores en la colestasis inducida por el ANIT, una serie de ratones fueron tratados con ANIT y se evaluó el efecto protector del HGF, estudiando las pruebas de función y daño hepático comúnmente usadas. La Figura 13 muestra el efecto inducido por el HGF. Los niveles de ALT (Figura 13A), AST (Figura 13B), ALP (Figura 13C), y GGT (Figura 13D) disminuyeron significativamente con respecto al control en 27.6, 7.4, 2.6 y 2.8 veces respectivamente, evidenciando el efecto protector del HGF. Por otro lado, se observó que el HGF induce una disminución en la bilirrubina total en 5.4 veces (Figura 13E), así como una disminución de 10.9 veces en la bilirrubina directa en el grupo co-tratado, comparado con los animales tratados solo con el ANIT (Figura 13F). De forma similar, el tratamiento con HGF normalizó completamente los niveles de sales biliares tanto en suero (Figura 13G), como en tejido hepático (Figura 13H), mejorando los niveles en 19.3 y 1.7 veces respectivamente.

Los resultados de las pruebas de función y daño hepático correlacionan con el aspecto macroscópico del hígado. Los animales tratados con HGF y ANIT muestran una notable mejoría con menos lesiones observadas en comparación con los animales tratados con ANIT (flechas, Figura 14A) y una recuperación en el color del órgano, con respecto a los animales tratados solo con el ANIT. Se observa igualmente recuperación en el tamaño del hígado, lo cual concuerda con el cociente del peso del

órgano *vs* el peso corporal del animal (Figura 14B). El HGF restaura estos parámetros a valores similares con el animal control o no tratado.

El análisis histológico por H&E revela datos consistentes con lo observado tanto macroscópicamente como en las pruebas de función hepática. Las zonas necróticas disminuyeron notablemente (asteriscos, Figura 14C) en los animales tratados con HGF+ANIT con respecto al grupo tratado solo con ANIT. Mejor aún, el infiltrado inflamatorio presente en los animales tratados con ANIT (flechas) no se encontró en los animales co-tratados, lo que nos permite afirmar un control total del efecto inflamatorio del ANIT, esto fuertemente asociado con el decremento en las zonas de necrosis.

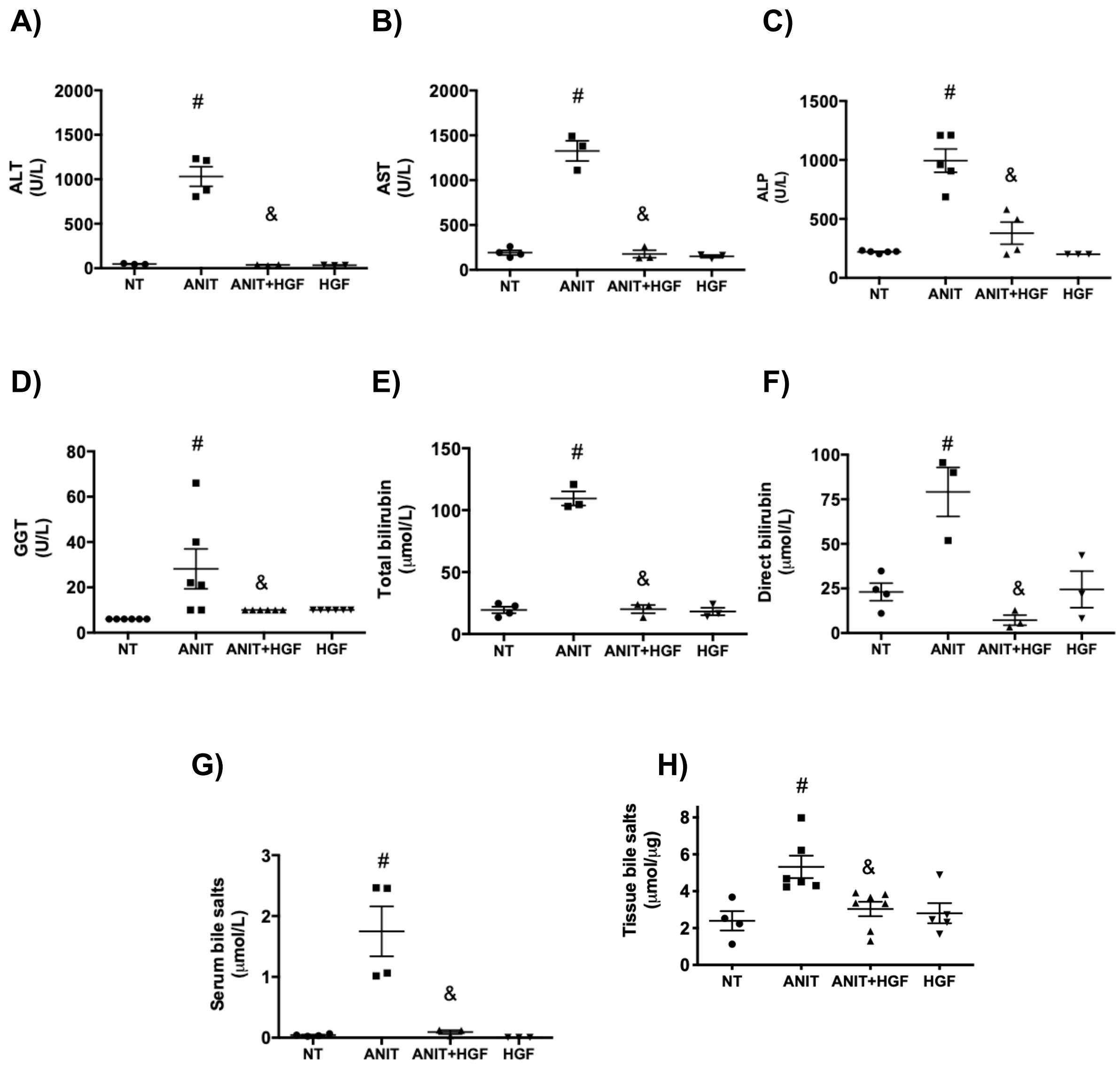



***Figura 13. HGF protege contra el daño colestásico inducido por ANIT.*** *A) ALT. B) AST. C) ALP. D) GGT. E) y F) Bilirrubina total y directa. G) y F) Contenido de sales biliares en suero y tejido.* #*p<0.01 vs NT;* &*p<0.01 vs ANIT.*

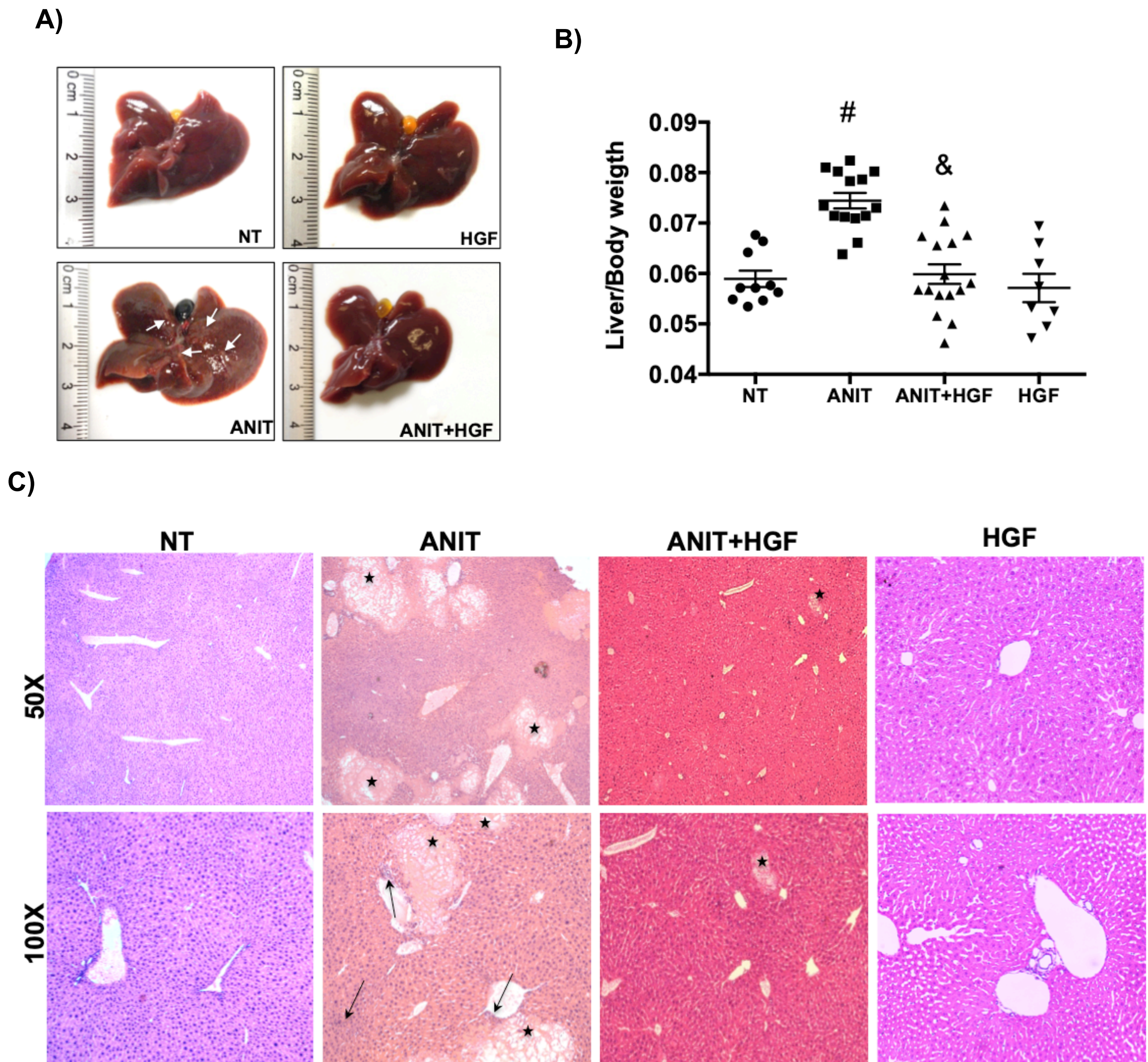


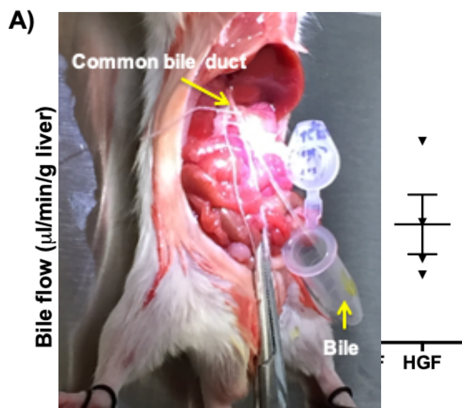

***Figura 14. HGF contrarresta el daño hepático tisular inducido por ANIT.*** *A) Inspección hepática macroscópica de los grupos. Las flechas blancas indican áreas necróticas. B) Cociente hígado-peso del ratón. C) Apariencia histológica por tinciones de H-E. Las estrellas negras indican las áreas con necrosis y las fechas muestran el infiltrado inflamatorio.* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

## El HGF restaura el flujo biliar afectado por el ANIT

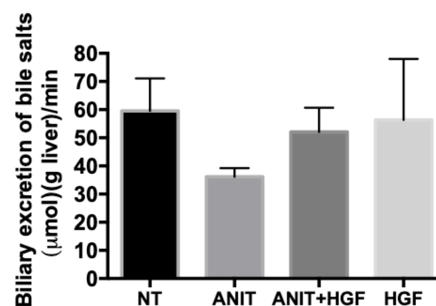
Un flujo biliar óptimo es fundamental para un buen funcionamiento del hígado y su influencia a nivel digestivo. En cambio, su disminución conduce a la retención de sustancias potencialmente tóxicas (sales biliares, bilirrubina), las que progresivamente dañan al hígado conduciendo a cirrosis y falla hepática grave. El flujo biliar disminuyó 1.7 veces con el tratamiento con ANIT comparado con el grupo NT, sin embargo, con el co-tratamiento, el flujo se vio restaurado a valores comparables con los normales (Figura 15A). Por otro lado, se observó una tendencia a incrementar la excreción de sales biliares (Figura 15B) que, si bien no llegó a ser estadísticamente significativa, puede tener relevancia fisiológica en contribuir parcialmente a la mejora histológica.

C)

A)




B)



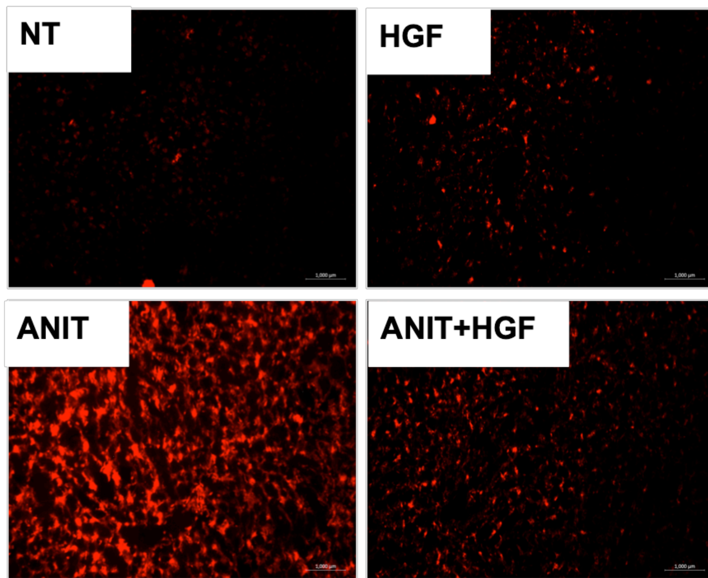

**El HGF disminuye la generación de ROS y el estrés oxidante.**

***Figura 15. HGF restaura el flujo biliar afectado por ANIT.*** *A) Flujo biliar. B) Excreción biliar de sales biliares. C) Canulación del ducto biliar.* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

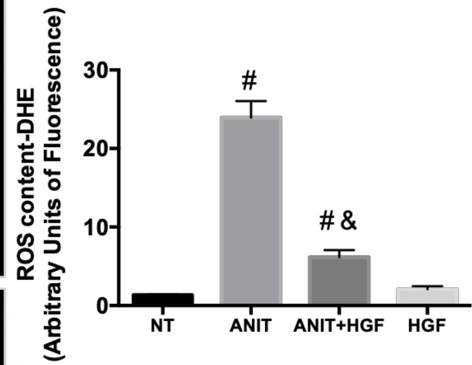
La colestasis está relacionada con fenómenos de estrés oxidante, ya sea como causa primaria de la misma o secundaria, como consecuencia de la retención de compuestos pro-oxidantes (por ej., sales biliares, bilirrubina) (Roma and Sanchez Pozzi 2008). Con el objetivo de determinar si el HGF contrarresta la colestasis controlando el estrés

oxidante inducido por el ANIT, se determinó el contenido del radical anión superóxido ($O_2^{\cdot -}$) en muestras frescas de tejido hepático de todos los grupos de tratamiento, empleando la sonda fluorescente DHE. Las imágenes de microscopía confocal (Figura 16A) muestran que el ANIT incrementó el contenido de $O_2^{\cdot -}$ 20 veces con respecto al grupo NT (Figura 16B). El co-tratamiento con HGF disminuyó la generación de este tipo de ROS. Este resultado es acorde con lo observado en el daño oxidante en lípidos conducente a la generación de lipoperóxidos (Figura 16C), el cual también fue disminuido significativamente por el HGF, llegando a niveles comparables con el grupo control. Este efecto protector puede estar mediado, en gran medida, por la activación del factor de transcripción Nrf2, el cual es altamente responsivo a la señalización del HGF en hepatocitos (Clavijo-Cornejo, Enriquez-Cortina et al. 2013). La Figura 16D muestra cómo el HGF es capaz de activar a Nrf2 sugiriendo que este podría ser un mediador de la protección inducida por el factor de crecimiento, en línea con datos previamente reportados por nuestro grupo (Clavijo-Cornejo, Enriquez-Cortina et al. 2013, Gomez-Quiroz et al. 2016).

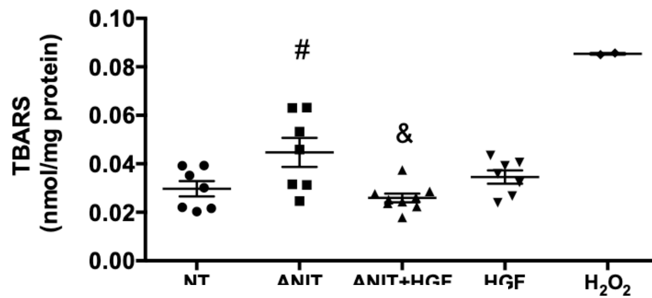
A)




B)




C)




D)

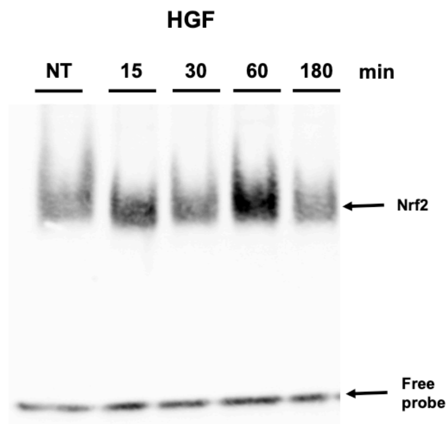


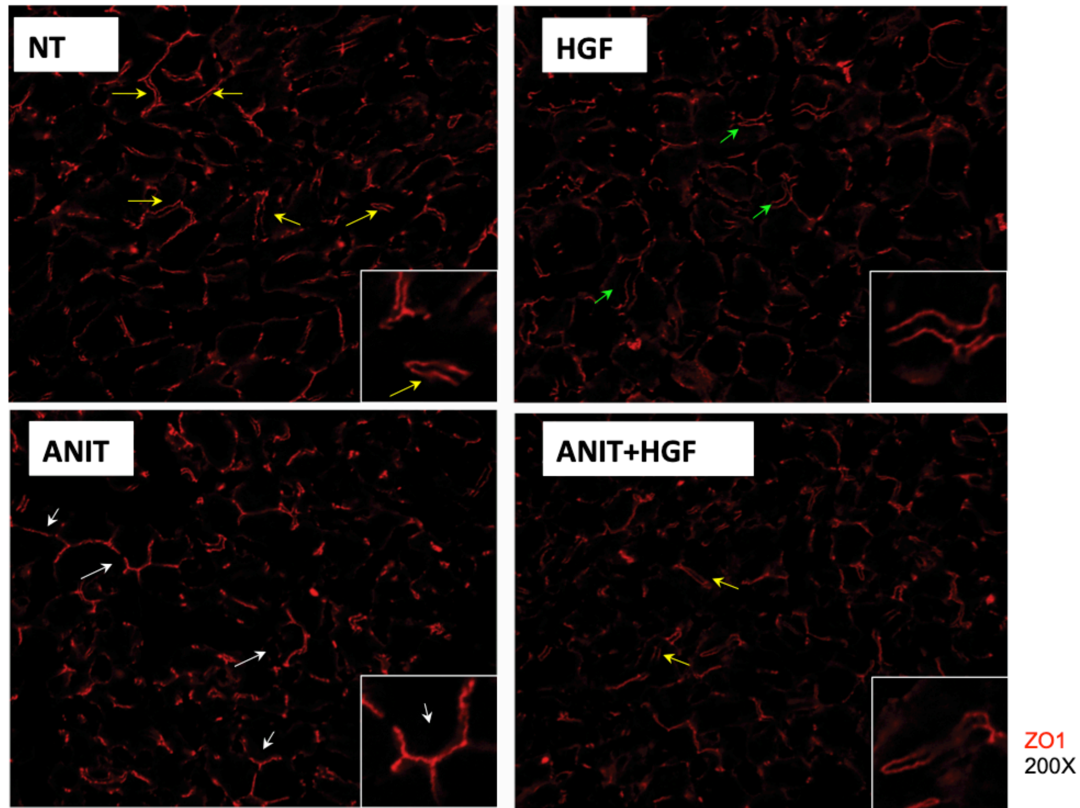

***Figura 16. HGF contrarresta las alteraciones en el balance redox inducidas por ANIT.*** *A) Contenido de anión superóxido en tejido hepático. B) Análisis densitométrico del contenido de anión superóxido, C) Oxidación de lípidos, medidos a través de la generación de TBARS. D) EMSA para Nrf2.* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

**El HGF preserva la estructura de las uniones estrechas (tight junction, TJ) afectadas por el ANIT.**

Para seguir con la exploración de los efectos anticolestásicos desplegados por el HGF, y dado que la integridad de las TJ, que sellan el canalículo biliar, es fundamental para mantener las fuerzas impulsoras osmóticas que generan el flujo biliar (Boyer 1983), se analizó el efecto del HGF en la estructura de las TJ por la inmunolocalización de la proteína zonula occludens 1 (ZO-1), una proteína asociada a uniones estrechas que posibilita su marcación (Kubitz et al. 2005). Se procedió al estudio de posibles alteraciones estructurales de las TJ a través de la cuantificación de las estructuras canaliculares normales en forma de "doble riel" o "train track" (Yeh et al. 2010). La Figura 17A muestra las estructuras canaliculares en condiciones normales (grupo NT), donde se puede observar un espacio intercelular (hemicanales) formado por la oposición de dos hepatocitos, cuya estructura esta limitada por TJ, estructura identificada como de "doble riel" (Figura 17A, flecha amarilla, y subcuadro insertado). El tratamiento con ANIT indujo un significativo decremento en el número de estructuras de "doble riel" canalicular (Figura 17B). Por el contrario, el grupo tratado solo con ANIT mostró predominantemente características de "monorriel" (fechas blancas), mostrando estructuras en espiral (subcuadro insertado). El co-tratamiento con HGF preservó eficientemente la estructura de "doble riel", de manera de alcanzar proporciones similares a las de los animales no tratados. Sugestivamente, el tratamiento solo con HGF incrementó substancialmente el ancho canalicular (Figura 17A, flechas verdes) comparado con el grupo NT (1.4 veces) y normalizó el decremento inducido por el ANIT en este parámetro (Figura 17C).

Con la finalidad de corroborar el rol central que tiene el HGF/c-Met en la génesis y conservación de la estructura canalicular, decidimos explorar las características que guardan las estructuras canaliculares en ratones con eliminación condicional en hígado de c-Met (Gomez-Quiroz, Factor et al. 2008), lo que en teoría debería tener un fenotipo similar al observado en nuestros animales tratados con ANIT, quienes comparten con los ratones deficientes en c-Met un estado redox pro-oxidante. El tejido hepático de ratones c-MetKO muestra las mismas anormalidades observadas en los ratones tratados con ANIT (Figura 18A), esto es estructuras en "monorriel" y con canales en espiral (flecha verde), comparado con el animal WT que presenta estructuras predominantemente en "doble riel" (flecha amarilla). Curiosamente, este efecto observado en ratones c-Met KO no se relaciona con cambios en el contenido de ZO-1 (Figura 18B). Estos datos sugieren una influencia directa del HGF/c-Met en la organización de las TJ, las cuales son requeridas para una secreción normal de bilis.

A)




C)

C)

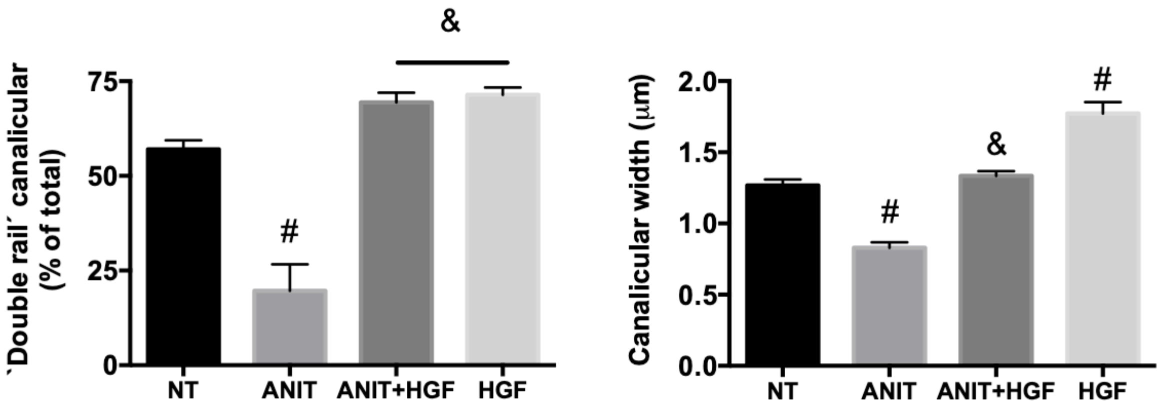
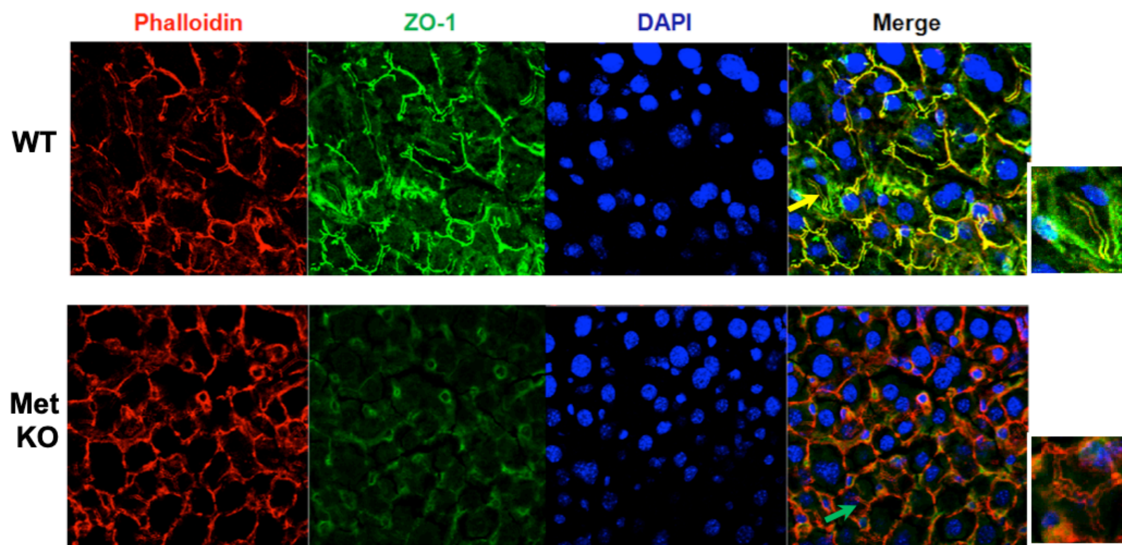



***Figura 17. HGF contrarresta las alteraciones en la estructura de las uniones estrechas intercelulares inducidas por ANIT.*** *A) Inmunofluorescencia de ZO-1. Las flechas amarillas, blancas y verdes muestran "doble riel", "monorrieles" y "doble rieles" más anchos, respectivamente. B) Cuantificación de estructuras de tipo "doble riel". C) Cuantificación del ancho canalicular,* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

A)




B)

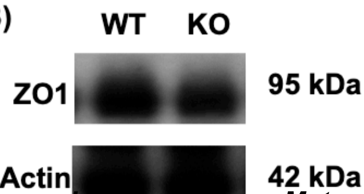



***Figura 18. La presencia del receptor c-Met es esencial para la estructura de las uniones estrechas.*** *A) Inmunoflurescencia de ZO-1 (marcador de uniones estrechas intercelulares) y de faloidina (marcado de actina pericanalicular) en rarones KO para c-Met. B) Western blot de ZO-1.*

**El HGF induce la expresión de transportadores basolaterales de la familia ABC**

Los cambios en la expresión de transportadores de la familia ABC canaliculares y basolaterales juegan un rol importante en la génesis y en la respuesta adaptativa de los procesos colestásicos, al ser los principales determinantes de la depuración biliar y renal de compuestos colefílicos, respectivamente (Roma, Crocenzi et al. 2008). Por esta razón, se exploraron la expresión de los genes *Abcc2*, *Abcb11*, *Abcc3*, y *Abcc4* y el contenido tisular de sus productos proteicos Mrp2, Bsep, Mrp3, y Mrp4, respectivamente. *Abcb11*/Bsep (Figura 19A) es un transportador canalicular que exporta sales biliares hacia el lumen canalicular, mientras que *Abcc2*/Mrp2 (Figura 19B) excreta endo y xenobióticos glutationalizados o glucoronizados, como por ejemplo los mono y diglucurónidos de bilirrubina. La expresión génica de ambos transportadores incrementó con el tratamiento con ANIT, posiblemente como un mecanismo de adaptación en colestasis, mientras que el co-tratamiento con HGF contrarrestó estos cambios. Por otro lado, las expresiones génicas de los transportadores basolaterales *Abcc3*/Mrp3 y *Abcc4/*Mrp4 incrementaron con el co-tratamiento con el factor de crecimiento (Figura 19C y D, respectivamente). Relevantemente, la expresión génica de *Abcc4/*Mrp4 incrementó notablemente luego del tratamiento con el factor de crecimiento administrado individualmente. El contenido protéico de estos transportadores presentó efectos diferentes al que sufrieron los mensajeros (Figura 20). Solo *Abcc2*/Mrp2, pero no *Abcb11/*Bsep, incrementó sus niveles proteicos por efecto del ANIT administrado *per se*. Por su parte, el efecto detrimental de ANIT *per se* sobre el transportador *Abcc4/*Mrp4 a nivel de proteína no se explicó por causas transcripcionales, sugiriendo más bien mecanismos post-

traduccionales de degradación (Figura 20). Las mejoras de los niveles de mensajeros para Mrp3 y Mrp4 en el grupo co-tratado con HGF solo fue reproducida a nivel de proteína para *Abcc4/*Mrp4 (Figura 20), pero el grupo ANIT+HGF presentó niveles proteicos de *Abcc3/*Mrp3 superiores al grupo control (Figura 20). Tomados de conjunto, el grupo co-tratado con HGF mostró siempre niveles proteicos mayores de los transportadores de eflujo basolateral respecto del grupo tratado solo con ANIT, demostrando que el factor de crecimiento puede mejorar la depuración alternativa de sales biliares por vía renal, previa extrusión de esos compuestos a través de la membrana basolateral.

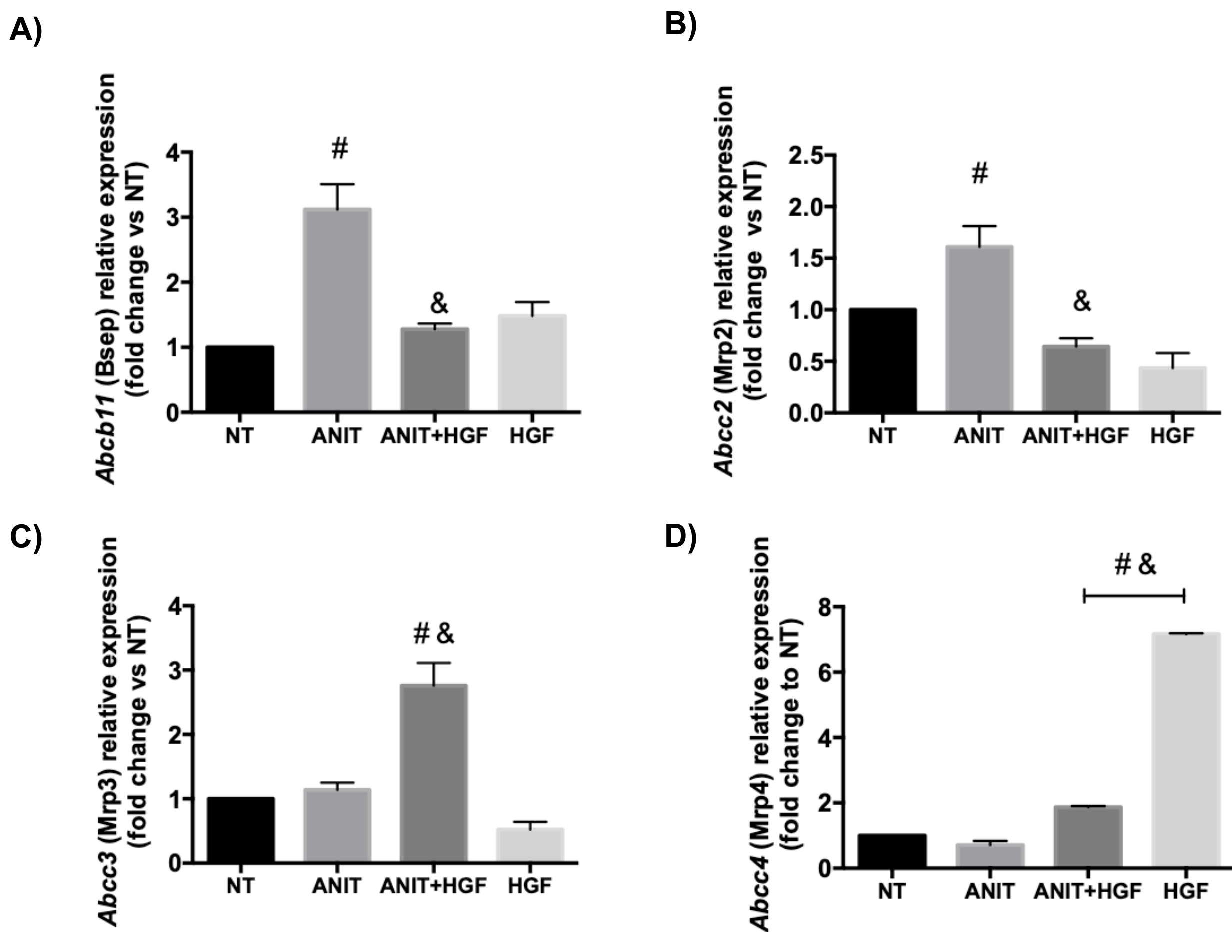
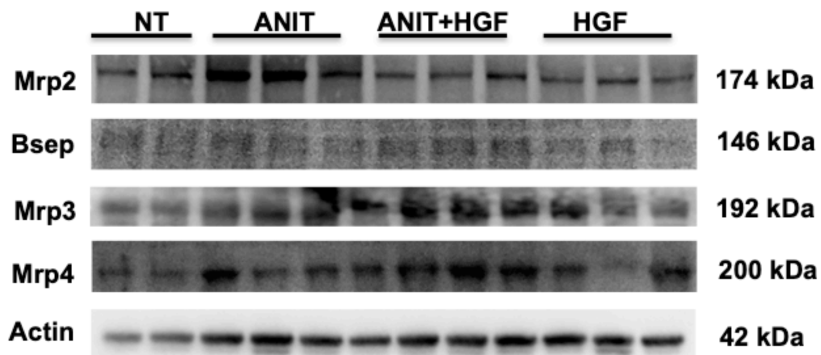
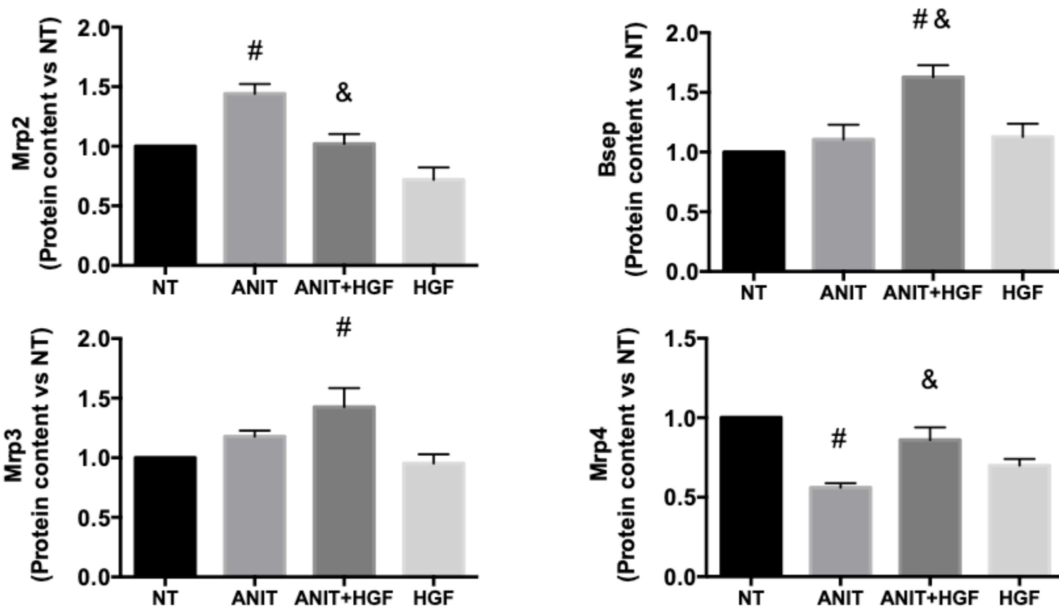



***Figura 19. HGF induce cambios en la expresión génica de transportadores de eflujo canaliculares y basolaterales****. Niveles de expresión génica del mensajero de A) Abcc1 (Bsep). B) Abcc2 (Mrp2). C) Abcc3 (Mrp4). D) Abcc4 (Mrp4). #p<0.01 vs NT; &p<0.01 vs ANIT.*




## El HGF normaliza el contenido de CFTR abrogado por el ANIT

***Figura 20. Cambios en el contenido protéico de tránsportadores ABC de eflujo canaliculares y basolaterales.*** *#p<0.01 vs NT; &p<0.01 vs ANIT.*

Una característica común en las colangiopatías es la secreción defectuosa ductular de un fluido rico en carbonato, la cual es impulsada por un canal de cloruro dependiente de ATP, el CFTR (Melero et al. 2002). Por ser este considerado un factor crítico en la protección del epitelio biliar en las enfermedades colestásicas, decidimos explorar el

estado que guarda esta proteína de membrana apical. La Figura 21 muestra el contenido de CFTR en tejido hepático, determinado por Western blot. Se observa un decremento del contenido proteico del CFTR de un 60% en los ratones tratados con ANIT, efecto que fue recuperado a valores control en los ratones co-tratados con HGF.

**El HGF protege el cilio primario colangiocelular del daño inducido por el ANIT**

El cilio primario del colangiocito es de suma importancia en la secreción biliar ductular normal, por lo que, si presenta defectos, impacta negativamente en la fisiología de los colangiocitos (Mansini et al. 2018). Se realizó una serie de observaciones por medio de microscopía electrónica de barrido (SEM) de tejido hepático proveniente de animales de los cuatro grupos de estudio, en busca de cambios morfológicos en el cilio primario. La Figura 22 muestra que el ANIT indujo un acortamiento importante en el cilio (flechas amarillas), mientras que el co-tratamiento con HGF contrarrestó dicho cambio morfológico.

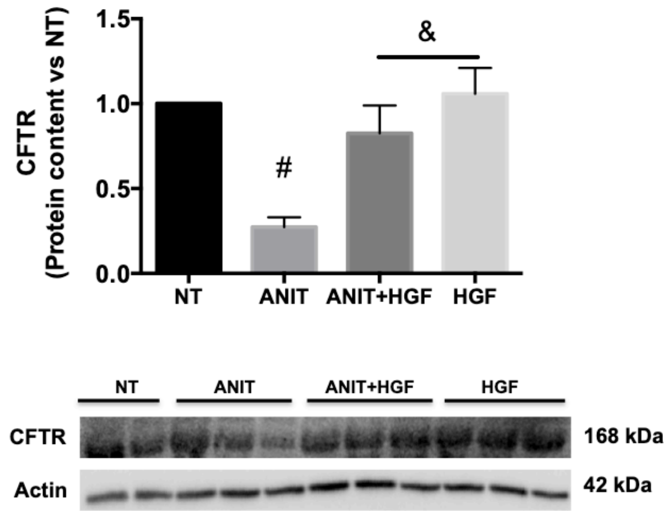



***Figura 21. HGF restablece el contenido de CFTR.*** *#p<0.01 vs NT; &p<0.01 vs ANIT.*

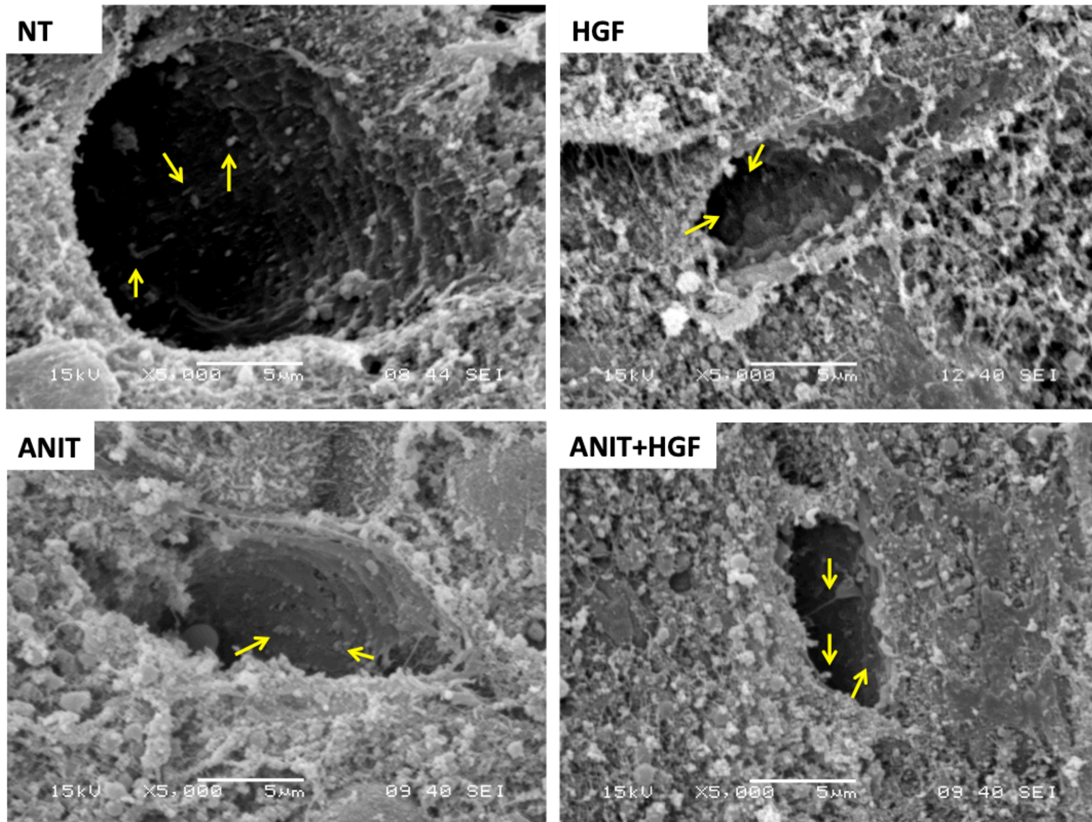



***Figura 22. HGF preserva el cilio primario colangiocelular.***

**El HGF contrarresta los efectos deletéreos de la colestasis a nivel renal**

Es bien conocido que en las enfermedades hepáticas, particularmente las colestásicas, se compromete notablemente la función renal (Terg et al. 2009), lo que eventualmente conlleva al desarrollo del síndrome hepatorrenal o, alternativamente, de la nefropatía colémica, esta última causada por un incremento en la circulación sistémica de ácidos biliares (Fickert, Krones et al. 2013).

En la Figura 23A, se puede observar claramente coluria (cambio en la coloración de la orina producido por la excreción urinaria de bilirrubina) al final de tratamiento con ANIT, mientras que el tratamiento con HGF restaura este marcador, en concordancia con la normalización de los niveles séricos de bilirrubina que HGF produce en los animales intoxicados con ANIT (Figura 13E y F). Para extender este concepto a otros compuestos retenidos sistémicamente por la colestasis con ANIT, se determinó el contenido de sales biliares en orina (Figura 23B), mostrando que el ANIT lo incrementa significativamente, mientras que el HGF lo regresa a valores control; esto está nuevamente en concordancia con la normalización de los niveles séricos de sales biliares que produce HGF (Figura 10B). La acumulación de creatinina en sueron es un indicador de disfunción renal. Como consecuencia del tratamiento con ANIT se observó un incremento importante en los niveles de creatinina sérica, mientras que el tratamiento con HGF la regresa a valores normales (Figura 23C). La depuración de creatinina, el cual es un parámetro de función renal, se vio seriamente afectada en los animales tratados con el ANIT, mientras que el co-tratamiento con HGF mejoró notablemente la depuración (Figura 23D).

Con la finalidad de tener certeza del efecto protector del HGF sobre el daño renal, se determinó la albuminuria en los animales de experimentación, un parámetro indicativo de daño glomerular o del epitelio tubular (Eaton D. 2004). La Figura 24A muestra que el tratamiento con ANIT genera albuminuria, mientras que el HGF lleva a valores control la presencia de esa proteína en orina. La proteína HSP72 se ha reportado que es un buen marcador temprano y sensible para la detección de daño tubular agudo en riñón (Barrera-Chimal, Perez-Villalva et al. 2011). La Figura 24B muestra un Western blot de la proteína HSP72 en orina, mostrando un incremento importante de dicha proteína en los ratones tratados con ANIT, mientras que el co-tratamiento con HGF la lleva a valores indetectables, confirmando los efectos protectores del factor de crecimiento en el daño renal asociado a la colestasis. La excresión urinaria de ambúmina o HSP72 se normalizó en relación con la cantidad de creatinina excretada, mostrando así el incremento significativo de la excresión urinaria de albúmina y de HSP72 en los animales con ANIT, siendo este incremento contrarrestado por el co-tratamiento con HGF.

**El HGF preserva la histología renal en el daño inducido por ANIT**

Con la finalidad de tener una mejor evaluación del daño renal causado por la colestasis inducida por el ANIT, se realizó un análisis histológico de riñones de los animales bajo los distintos tratamientos. La Figura 25 muestra el aspecto normal de la estructura renal, con glomérulos bien definidos (G), túbulos distales y proximales (T) con una arquitectura normal, mostrando de una manera bien definida la luz de estas estructuras. El tratamiento con ANIT muestra dilatación de los túbulos, y la presencia

de cilindros tubulares (asterisco, “cast”, término en inglés). El co-tratamiento muestra claramente una recuperación en el tamaño del túbulo, así como en el número de cilindros por campo detectados.

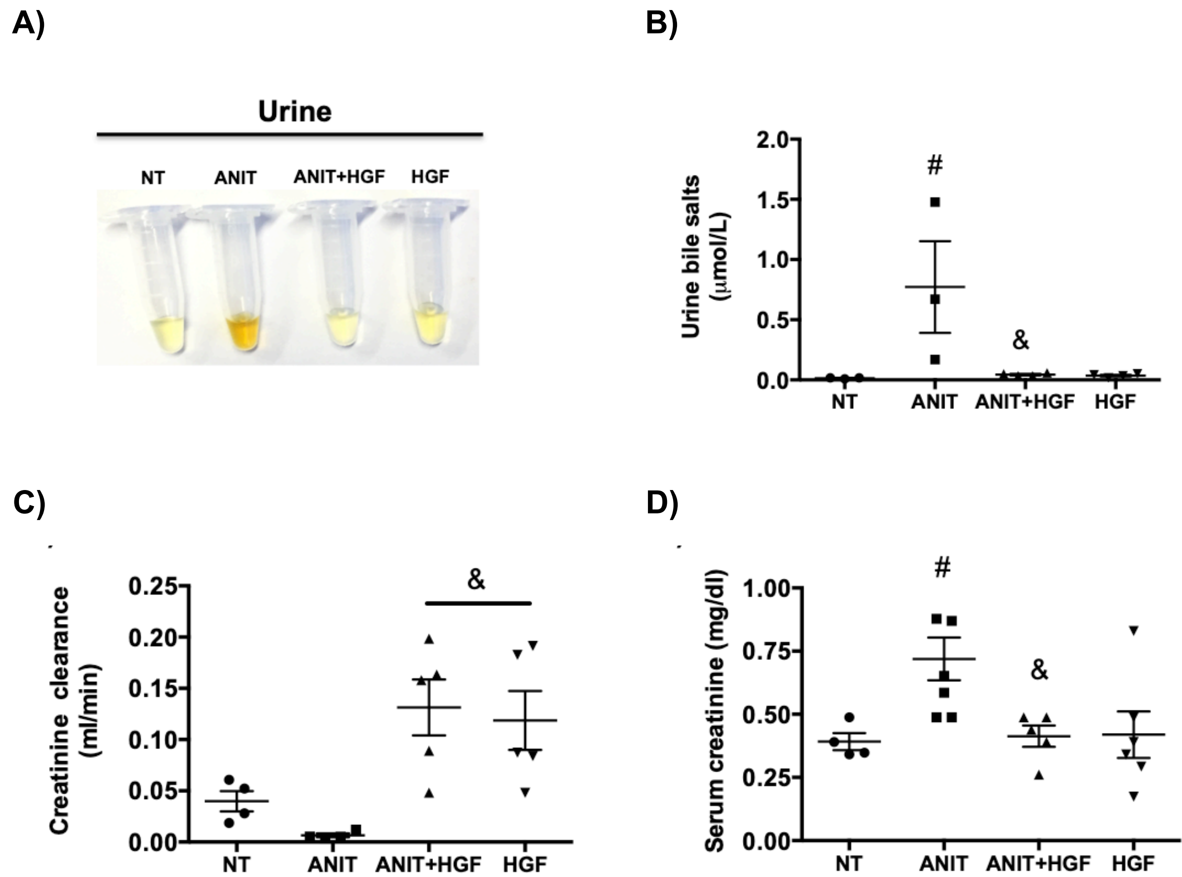
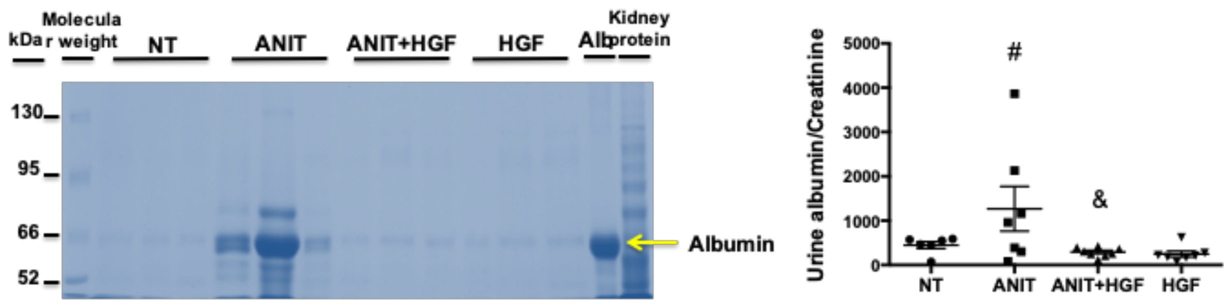



***Figura 23. HGF contrarresta los efectos de la colestasis a nivel renal.*** *A) Coloración urinario de los cuatro grupos. B) Contenido de sales biliares en orina. C) Depuración de creatinina. D) Contenido de creatinina en suer.* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

**A)**




**B)**

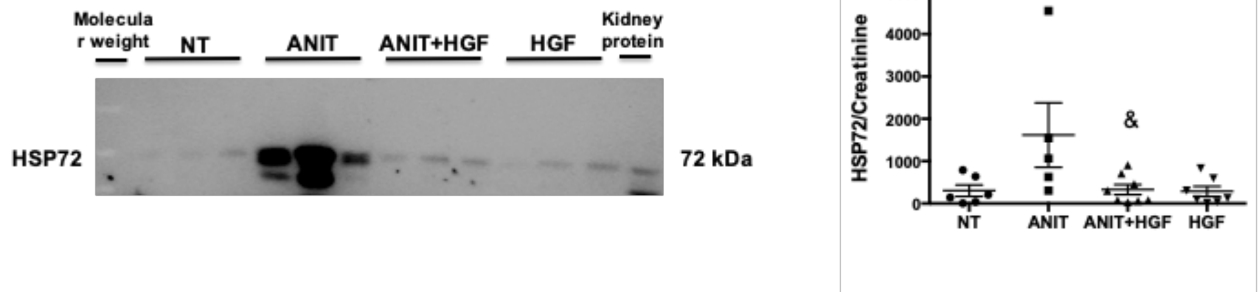



***Figura 24. HGF disminuye el daño tubular agudo en riñón.*** *A) y B) Contenido de albúmina y HSP72 en orina [#]p<0.01 vs NT; [&]p<0.01 vs ANIT.*

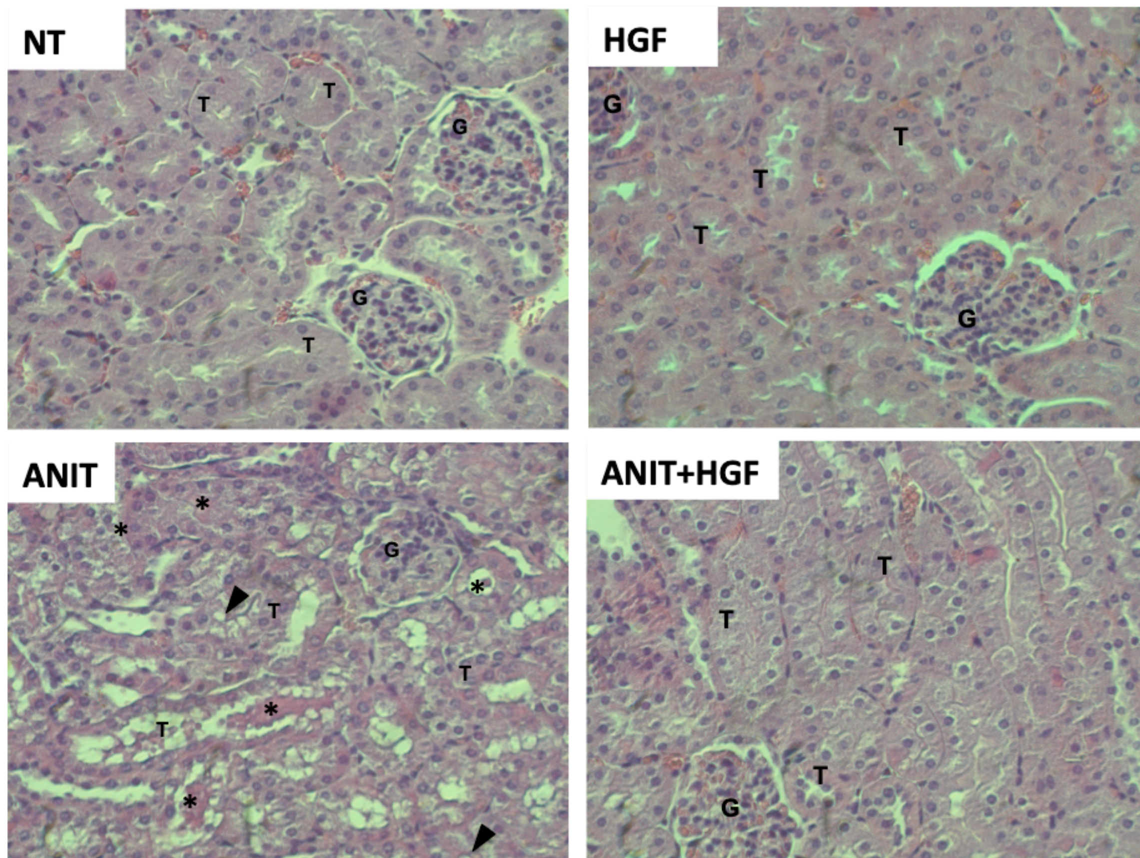



***Figura 25. HGF contrarresta el daño tubular inducido por ANIT.*** *G, glomérulo. T, túbulos proximales y distales. Asteriscos, "cast" tubulares y cabezas de flecha, vacuolas.*

## El HGF contrarresta el estrés oxidante renal inducido por la colestasis

Finalmente, se exploró la generación del anión superóxido en el tejido renal, buscando un efecto similar antioxidante del HGF al encontrado en el hígado. La Figura 26A muestra un incremento en la generación de esa ROS en los animales tratados con ANIT. Como se esperaba, el co-tratamiento con HGF disminuyó de manera significativa el contenido de anión superóxido. Este resultado correlaciona con la oxidación lipídica determinada por TBARS, ya que el HGF disminuye significativamente la lipoperoxidación (Figura 26B). Este resultado muestra que el mecanismo de daño es el mismo en riñón y en el hígado, esto es, la generación excesiva de ROS que conlleva al estrés oxidante y al daño celular.

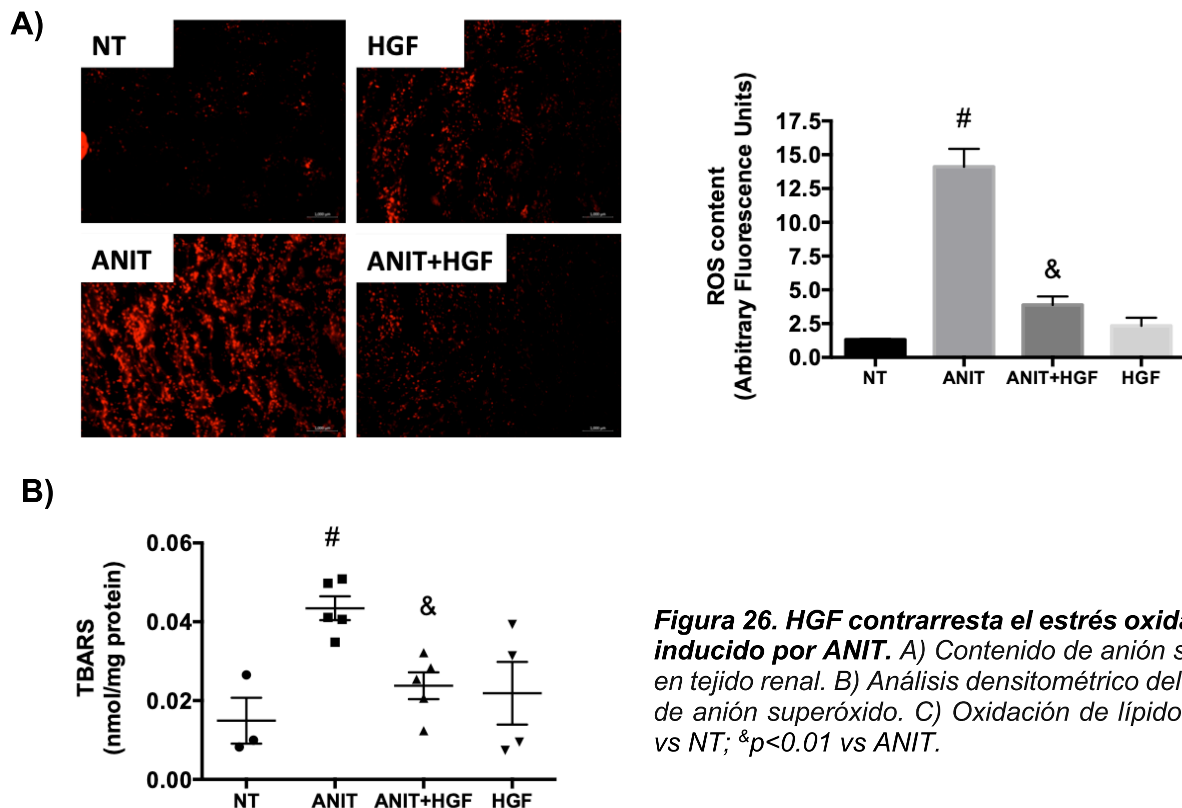



***Figura 26. HGF contrarresta el estrés oxidante renal inducido por ANIT.*** *A) Contenido de anión superóxido en tejido renal. B) Análisis densitométrico del contenido de anión superóxido. C) Oxidación de lípidos.* $^{\#}p<0.01$ *vs NT;* $^{\&}p<0.01$ *vs ANIT.*

## 8. Discusión

Las enfermedades hepáticas siguen representando un serio problema de salud pública, con un pronóstico pobre para los próximos años en gran medida debido al estilo de vida moderno, por lo que es necesario la búsqueda y caracterización de mecanismos de protección hepática que tiendan, eventualmente, al establecimiento de nuevos enfoques terapéuticos.

Se ha reportado extensivamente que el HGF ejerce efectos protectores en diversas enfermedades hepáticas, como la fibrosis (Marquardt, Seo et al. 2012), la enfermedad hepática alcohólica (Valdes-Arzate, Luna et al. 2009), la hepatocarcinogénesis (Takami et al. 2007), el daño inducido por fármacos (Enriquez-Cortina, Almonte-Becerril et al. 2013, Bello-Monroy et al. 2019), entre otros. Incluso, se ha reportado una mejora en la sobrevivencia celular en pancreatitis (Palestino-Dominguez et al. 2018) y protección pulmonar en tuberculosis (Bello-Monroy, Mata-Espinosa et al. 2019), en todos los casos por medio de la regulación del estado redox celular y control del estrés oxidante (Clavijo-Cornejo, Enriquez-Cortina et al. 2013). Sin embargo, poco se conoce de los efectos que despliega el HGF en la colestasis y como puede, eventualmente, ejercer efectos protectores en esta enfermedad.

La colestasis puede ser definida como un desorden que ocasiona defectos o impedimento en el flujo biliar normal desde el lumen canalicular de los hepatocitos hasta el duodeno (Del Valle Díaz et al. 2017), lo que ocasiona citotoxicidad por el exceso de sales biliares acumuladas en el órgano. El daño se presenta en todos los tipos celulares del hígado, pero, particularmente, en el colangiocito y el hepatocito, lo que ocasiona la enfermedad hepática.

La patogénesis de la colestasis permanece aún pobremente estudiada, a pesar del incremento en su incidencia (Salas-Silva et al. 2019), por lo que nuevas opciones terapéuticas deben ser desarrolladas para contrarrestar esta enfermedad.

En el presente trabajo, nos enfocamos en el estudio de los efectos protectores del HGF, empleando un modelo de colestasis en ratones inducida por el ANIT.

Este compuesto, altamente tóxico, induce colestasis conduciendo a la acumulación de sales biliares en el parénquima hepático. Si bien el mecanismo de la inducción de colestasis por ANIT no ha sido completamente elucidado, se tiene evidencia de que afecta la expresión de varios transportadores hepatobiliares involucrados en el manejo de sales biliares en un modelo de sándwich de hepatocitos primarios de rata (Guo et al. 2014). Los datos que presentamos en esta tesis soportan estos reportes, como se mostrará más adelante.

Las alteraciones producidas por ANIT en los transportadores hepatocelulares involucran la activación de AMPK dependiente de sales biliares, que utiliza la vía de señalización ERK1/2-LKB y la consecuente represión del receptor X de farnesilos (FXR), un regulador nuclear maestro de la homeostasis de sales biliares (Li et al. 2016). Tras el inicio del proceso colestásico, varios mecanismos de adaptación son establecidos con la finalidad de evitar un daño mayor. Entre otros, se expresan transportadores basolaterales que exportan sales hacia el torrente sanguíneo, permitiendo su excreción renal como una ruta alterna de depuración de estos compuestos tóxicos, lo cual puede eventualmente comprometer la integridad renal (Cui et al. 2009, Fickert, Krones et al. 2013).

Entre los mecanismos más importantes en el inicio y desarrollo de la colestasis se encuentra el estrés oxidante, particularmente en el modelo de ANIT, tal y como se confirma en el presente trabajo (Figura 16A y B). La lipoperoxidación, un marcador subrogado bien aceptado de daño por estrés oxidante, se incrementó rápidamente desde las 12 h de tratamiento con el ANIT, esto asociado a un incremento en la generación de ROS en el hígado (Figura 16C). Los mecanismos postulados como responsables del estrés oxidante inicial inducido por ANIT son poco conocidos. Un factor involucrado en el desbalance redox podría ser, por un lado, las ROS derivadas de la infiltración de neutrófilos en la zona de daño (Kongo et al. 1999) y a la activación de la NADPH oxidasa 4 (Zhao et al. 2013), y por otro lado, a defectos en los sistemas de protección antioxidante, ya que se han observado disminuciones en los niveles de superóxido dismutasa y catalasa (Ohta et al. 2006). El estrés oxidante puede representar, por lo tanto, el primer agresor que media el fenómeno colestásico inducido por ANIT en el parénquima. El estrés oxidante induce rápidamente efectos colestásicos, particularmente la desorganización del citoesqueleto de actina, seguido de una endocitosis o internalización de los transportadores canaliculares como Bsep (Perez et al. 2006), Mrp2 (Sekine et al. 2006) y la pérdida de integridad de las uniones estrechas (Perez, Milkiewicz et al. 2006). Esto deriva en una segunda agresión mediada por una acumulación intracelular rápida de sales biliares en el hepatocito, lo cual agrava el estrés oxidante, ya que se sabe que las sales biliares presentan marcadas propiedades pro-oxidantes que promueven daño mitocondrial con escape de ROS de ese organelo, y subsecuente muerte celular por apoptosis y necrosis (Sokol

et al. 2006), tal y como lo soporta los resultados presentados en este trabajo (Figura 14C).

Con base a lo anterior, no es de sorprenderse que los antioxidantes presenten efectos anti-colestásicos en la injuria inducida por ANIT, como ha sido observado en diversos trabajos, donde se han empleado como antioxidantes el $\alpha$-tocoferol (Ohta, Kongo-Nishimura et al. 2006), la paeoniflorin (Zhao, Zhou et al. 2013), SRT1720 (Yu et al. 2017) y la melatonina (Calvo et al. 2001), entre otros. Sugestivamente, varios de estos compuestos ejercen sus acciones anti-colestásicas y a la vez activan el factor de transcripción Nrf2, sugiriendo (auque no probando) una asociación causal entre ambos fenómenos. Nuestro grupo ha reportado que Nrf2 puede ser selectivamente activado por el HGF y que, de hecho, este factor de transcripción antioxidante se presenta como uno de los principales mecanismos de protección desplegado por este factor de crecimiento (Clavijo-Cornejo, Enriquez-Cortina et al. 2013); de hecho la Figura 16D, muestra en nuestro modelo animal, la activación de Nrf2 por el HGF, lo cual sustenta el concepto que el estrés oxidante es un factor clave en la colestasis inducida por ANIT y que Nrf2 juega un papel central en la protección (Roma and Sanchez Pozzi 2008). De ser así, esta sería la primera evidencia que un agente anticolestásico generara sus efectos protectores a través de un mecanismo puramente asociado a la activación de Nrf2. Por ejemplo, el oltipraz, un activador bien conocido y hasta recientemente creido selectivo para inducir Nrf2, presenta ciertos efectos anti-colestásicos en ratones Nrf2KO (Tanaka, Aleksunes et al. 2009). Esto sugiere dos aspectos relevantes, el primero que el oltipraz ejerce funciones anti-colestásicas por medio de una ruta

independiente de Nrf2, y, por otro lado, que Nrf2, si bien es importante en la protección que ejerce el oltipraz, no es la única ruta que la sustenta. Su efecto anticolestásico en ratones Nrf2KO puede deberse a una posible regulación del receptor constitutivo de androstona (CAR), un receptor nuclear que despliega efectos anti-colestásicos, y que es activado por oltipraz (Merrell et al. 2008).

El posible mecanismo por el cual el HGF media su efecto anti-colestásico vía Nrf2 es multifactorial. Nuestro grupo de investigación ha mostrado que el HGF es un regulador maestro del estado redox celular, ya que activa a Nrf2, en un proceso dependiente de ROS derivadas de la NADPH oxidasa (Clavijo-Cornejo, Enriquez-Cortina et al. 2013), lo que induce la producción de enzimas antioxidantes y la abrogación tardía de sistemas productores de ROS, como la misma NADPH oxidasa, la cual, como se indicó antes, se ha involucrado en el efecto colestásico del ANIT (Zhao, Zhou et al. 2013). Nuestros resultados muestran claramente una respuesta antioxidante de HGF (Figura 16), lo cual reafirma que el estrés oxidante es el primer agresor en el modelo colestásico mediado por ANIT.

Además de la inducción de proteínas antioxidantes mediada por Nrf2, el HGF induce también proteínas de fase II y III de destoxificación (Clavijo-Cornejo, Enriquez-Cortina et al. 2013), siendo este aspecto, tal vez, incluso más relevante que la protección antioxidante, aunque no excluyente.

En cuanto a la inducción de proteínas antioxidantes de fase II por Nrf2, a las ya mencionadas catalasa y superóxido dismutasa, deben sumarse numerosas enzimas que directa o indirectamente cumplen funciones antioxidantes, tal como la γ-glutamilcisteína sintasa (involucrada en la síntesis de glutatión), la glutatión peroxidasa

(involucrada en la depuración de peróxidos mediada por glutatión), la glutatión reductasa (involucrada en la regeneración de glutatión reducido), la glutatión *S*-transferasa (involucrada en la conjugación con glutatión de lipoperóxidos y otros compuestos pro-oxidantes para su posterior depuración biliar), la hemo oxigenasa 1 (enzima productora del poderoso antioxidante bilirrubina) y enzimas del sistema de la tioredoxina, involucrado en la detoxificación de peróxidos, como la tioredoxina, la peroxiredoxina, y la tioredoxina reductasa, entre otras (Zhang et al. 2013).

Por su parte, la expresión de proteínas de fase III, como los transportadores biliares, ejercen funciones de eliminación de sustancias tóxicas, ejerciendo, de cierta forma, un efecto antioxidante indirecto al eliminar al inductor de la generación de ROS o de productos de oxidación que catalizan reacciones en cadena pro-oxidantes, como los lipoperóxidos (Weerachayaphorn, Mennone et al. 2012). Nrf2 puede modular positivamente los transportadores hepatocelulares cuya expresión es alterada por el ANIT, a la vez que induce bombas de exportación basolaterales para mantener bajos los niveles intracelulares de sales biliares a pesar de la falla secretora, lo cual abroga el segundo insulto asociado a la toxicidad de las mismas, principal responsable de la muerte hepatocelular. En el presente trabajo, se encontró un incremento en los transportadores basolaterales Mrp3 y Mrp4 debido al tratamiento con HGF (Figuras 19C y D y 20) en los animales co-tratados con ANIT. Estudios realizados en animales KO de Mrp3 y Mrp4 muestran que la bilirrubina conjugada (glucoronizada) es el principal sustrato para Mrp3, mientras que los ácidos biliares conjugados son preferentemente secretados por Mrp4 en el hígado colestásico (Mennone et al. 2006). Las sales biliares (Hofmann 1999) y la bilirrubina (Bellarosa et al. 2009), esta última

particularmente en su forma apolar no conjugada, representan sustancias altamente tóxicas que median la muerte celular por apoptosis o necrosis por medio de mecanismos diferenciados pero que tiene como centro de control a la mitocondria disfuncional, lo cual genera e incrementa el estrés oxidante, desorganizando la membrana plasmática, y conduciendo al estrés del retículo endoplásmico, entre otros efectos deletéreos; de hecho, el estrés del retículo endoplasmático sería un patomecanismo temprano en la colestasis por ANIT, visible a tan solo 12 h de la administración del compuesto, el cual puede explicar en parte la apoptosis hepatocelular que ocurre en este modelo (Yao et al. 2016). Incidentalmente, este estudio también encontró que ANIT perturba la proliferación del hepatocito y por ende la regeneración hepática necesaria para recuperarse de la injuria colestásica, por lo que las propiedades regenerativas del HGF podrían ser particularmente beneficiosas para reactivar este proceso reparativo.

Además de facilitar la exportación basolateral de sales biliares y bilirrubina posibilitando su depuración alternativa urinaria, el HGF ejerce efectos diferentes sobre los transportadores ABC canaliculares Bsep y Mrp2 a los que ejerce sobre los basolaterales, disminuyendo sus niveles a nivel transcripcional (Figura 19A y B). Esto refuerza el redireccionamiento de las sales biliares y la bilirrubina conjugada al torrente sanguíneo para su excreción urinaria en vez de biliar (Zollner et al. 2006). Dado que ANIT, una vez conjugado con glutatión, es excretado al lumen biliar vía Mrp2, y que es en el lúmen biliar, una vez disociado del glutatión, desde donde ejerce sus efectos tóxicos sobre el colangiocito, nuestro hallazgo que HGF no fue capaz de recuperar los niveles de Mrp2 reducidos por ANIT debe verse como un factor beneficioso y no

detrimental. Lo anterior es consistente con los hallazgos en ratas deficientes en Mrp2, las cuales presentan protección contra la colestasis inducida por ANIT (Dietrich et al. 2001). Es claro que la eliminación basolateral en lugar de biliar de ANIT evita el daño colestásico que se observa tanto en el modelo de la rata deficiente de Mrp2 como en nuestro modelo de HGF.

La falla en la inducción de los transportadores canaliculares Bsep y Mrp2, responsables de las fracciones del flujo biliar dependiente e independiente de sales biliares, respectivamente, resulta en la ausencia de un efecto colerético del HGF en los animales colestásicos, lo cual puede tener también implicaciones terapéuticas positivas, particularmente bajo condiciones de obstrucción biliar, como ocurre en el modelo que hemos usado. Esto también potencialmente aplica a las colangiopatías obstructivas en humanos, como la colangitis biliar primaria en estadios avanzados y la colangitis esclerosante primaria. Un ejemplo de los riesgos de inducir coleresis en nuestro modelo (y en otras colangiopatías obstructivas experimentales y clínicas) lo aporta el agente colerético ácido ursodeoxicólico (UDCA), el fármaco más ampliamente usado en el tratamiento de la mayoría de las hepatopatías colestásicas. UDCA induce selectivamente a Bsep para incrementar el flujo de bilis y la excreción de sales biliares hacia la ruta biliar en el modelo de ANIT, agravando el daño cuando el ducto biliar esta obstruido (Zhang et al. 2018). Esto ocurre como consecuencia de infartos biliares del canal de Hearing debido al incremento de la presión hidrostática intraluminal en los conductos obstruidos, lo cual permite la extravasación y contacto directo de la bilis con los hepatocitos, resultando en citotoxidad por los altos niveles de sales biliares que contiene (Fickert et al. 2013). Esto podría explicar por qué la

terapéutica con UDCA puede tener ciertos beneficios en el tratamiento de las colangiopatías obstructivas solo en etapas iniciales de su historia natural, donde el proceso obstructivo aún no es prominente (Roma et al. 2011). Por lo tanto, las opciones terapéuticas con agentes farmacológicos que tiendan a redirigir los compuestos biliares tóxicos hacia la orina sin generar ni coleresis ni incremento en la excreción biliar de sales biliares, como lo hace el HGF (Figuras 13 y 23), pueden ser particularmente útiles en las colangiopatías obstructivas.

El HGF pareciera tener efectos favorables no solo a nivel funcional en el daño colestásico, sino también en aspectos estructurales importantes, como es el caso de la integridad de las uniones estrechas interhepatocitarias. Se sabe que el ANIT altera funcionalmente la permeabilidad paracelular dependiente de las uniones estrechas en tiempos tan cortos como 16 h de tratamiento (Lowe et al. 1985, Kossor, Meunier et al. 1993), permitiendo la disipación de los gradientes osmóticos biliares vía derivaciones paracelulares y, consecuentemente, impidiendo la generación osmótica del flujo biliar. En este trabajo de tesis, se demuestra que estos cambios funcionales en la función de barrera de las uniones estrechas tienen una clara correlación estructural. ZO-1, una proteína asociada a las uniones estrechas que une filamentos de actina a las TJ, pierde su característica estructural de arreglo en “doble riel” (Figura 17A y B). Se ha reportado una reducción en la expresión de actina por efecto del tratamiento con ANIT a las 48 h (Hua LI et al. 2016), por lo que la desorganización de ZO-1 puede ser una consecuencia directa de la pérdida de su proteína de anclaje, con la cosecuente alteración de la organización normal de las TJ. Esto no elimina la posibilidad de que, además de una disminución de expresión, puede haber ocurrido una desorganización

de la F-actina, un fenómeno común en condiciones de estrés oxidante (Perez, Milkiewicz et al. 2006), lo cual puede también haber contribuido a la desorganización de ZO-1. Nuestros resultados muestran que el HGF preserva la integridad de ZO-1 (Figura 17), abriendo la posibilidad de que el HGF también prevenga los desórdenes funcionales en las TJ.

Con la finalidad de observar la función normal de HGF/c-Met a nivel estructural en las uniones estrechas, realizamos una IF de ZO-1 y actina en estos animales, mostrando disturbios muy similares a los encontrados en nuestro tratamiento con ANIT. En efecto, el fenotipo de "doble riel" se vio seriamente afectado, con la aparición de estructuras en espiral (Figura 17A y B), que sugieren fuertemente impedimento del flujo biliar. Este patrón de cambio estructural es muy similar al que presentan los ratones KO para β-catenina, un componente clave de las uniones adherentes que mantiene a las caderinas en dichas estructuras para generar la adhesión intercelular (Yeh, Krauland et al. 2010). La proteína ZO-1 está unida físicamente tanto a las uniones estrechas (vía ocludina) como a las uniones adherentes (vía β-catenina), por lo que puede ser igualmente desorganizada ante alteraciones en ambas moléculas (Campbell et al. 2017). Esto explica el patrón similar de cambio en la disposición de ZO-1 entre los animales tratados con ANIT y los animales KO para β-catenina, ya que su anclaje a estructuras de sostén estaría igualmente comprometido, ya sea por alteraciones en ocludina o actina (en la colestasis por ANIT) o alteraciones en la β-catenina (en los animales KO para esta molécula).

Los datos obtenidos en esta investigación doctoral también proveen evidencia que existe daño a nivel de la integridad y función del colangiocito que son atenuadas, o incluso normalizadas, por el HGF. Las alteraciones en la integridad del colangiocito son sustentadas por los cambios observados en los niveles séricos de GGT (Figura 13), un marcador bioquímico selectivo de daño colangiocelular que se induce y libera de la membrana plasmática del colangiocito por la acción detergente de sales biliares, quienes alteran su integridad desde el lado luminal (Bulle et al. 1990). Las alteraciones funcionales del colangiocito inducidas por ANIT comprenden el decremento en el contenido del canal del cloruro dependiente de ATP CFTR, el cual se expresa exclusivamente en los colangiocitos e impulsa la secreción acuosa alcalina ductular (Figura 21) y, finalmente, el acortamiento de los cilios primarios característicos de este tipo celular, y que modulan algunas de sus funciones (Figura 22).

El daño al conducto biliar por el ANIT ocurre por la formación reversible de ANIT-GSH, el cual es muy inestable, y se puede disociar parcialmente una vez secretado a la vía biliar por Mrp2 por la alcalinidad del medio, derivando en GSH y el altamente tóxico ANIT no conjugado (Dietrich, Ottenhoff et al. 2001). El efecto tóxico parece ser mediado por los productos altamente reactivos formados del isotiocianato que contiene la molécula después de la disociación del GSH (Cullen et al. 2010), que pueden interactuar con los grupos sulfidrilos de proteínas de la superficie colangiocelular. Las proteínas dañadas son endocitadas y transportadas al retículo, donde pueden iniciar un estrés por proteínas mal ensambladas y estrés oxidante. (Cullen, Falls et al. 2010). En el efecto protector del HGF a este nivel, puede estar involucrada la exportación acelerada de ANIT-GSH hacia la sangre vía la inducción de Mrp4, el cual es un

eficiente transportador de especies glutationalizadas (Bai et al. 2004), y su posterior depuración por vía urinaria. También pueden estar involucrados efectos antioxidantes del HGF a nivel del colangiocito, donde pudo haber inducido enzimas antioxidantes vía Nrf2, incluida la γ-glutamilcisteína sinetasa, enzima clave en la formación de GSH (Valdes-Arzate, Luna et al. 2009). Esta posibilidad es sustentada por el hecho que los colangiocitos expresan al receptor de HFG c-Met (Menakongka et al. 2010). El hallazgo de que el CFTR es reprimido en los animales tratados con ANIT y que su expresión es restaurada por HGF son aportes nuevos que pueden tener profundas implicaciones funcionales y terapéuticas. Es sabido que el ANIT induce una fuerte respuesta inflamatoria y que estos mediadores pueden afectar la expresión del CFTR en epitelios (Besancon et al. 1994), probablemente por mecanismos asociados a generación de ROS (Qu et al. 2009). La afectación del CFTR por el ANIT puede contribuir al mal funcionamiento del mecanismo de coleresis ductular dependiente de bicarbonato, debido a la ausencia de cloruro luminal, que debería ser intercambiado por bicarbonato vía el intercambiador AE2 para permitir la secreción de bicarbonato y la consiguiente generación de la secreción acuosa-alcalina ductular. Esto puede producir: *i*) decremento en el flujo biliar ductular, permitiendo la formación de tapones mucosos, agravando el proceso obstructivo y, *ii*) atenuación del efecto conocido como “sombrilla de bicarbonato”, el cual mantienen las sales biliares en su forma iónica no difusible, impidiendo que el colangiocito sea atacado por estos compuestos tóxicos (Freudenberg et al. 2008). Aunado a lo anterior, la pérdida del cilio primario en colangiocitos, como lo demostramos con la SEM, puede también contribuir a la patogénesis. Los cilios primarios son quimiosensores que median la respuesta de

adaptación disparada por las sales biliares en el colangiocito, ya que expresan el receptor TGR5 de sales biliares (Keitel et al. 2010). El HGF preserva al cilio, y de esta forma, el colangiocito podría contrarrestar el daño ocasionado por las sales biliares induciendo una respuesta de adaptación.

Si bien los efectos anticolestásicos del HGF fueron inducidos aquí por dosis suprafisiológicas (o sea, farmacológicas) de HGF, nuestro grupo ha reportado efectos anticolestásicos del HGF endógeno a niveles fisiológicos. En efecto, al ser sometidos a una dieta alta en colesterol, los ratones MetKO carentes de capacidad de transducción de la señalización del HGF presentan hiperbilirrubinemia y aumento en los niveles séricos de la enzima de colestasis ALP, asociado a un decremento en la expresión de FXR, un factor de transcripción que juega un papel fundamental en la homeostasis de sales biliares (Gomez-Quiroz, Seo et al. 2016). Esto indica que, aún a concentraciones fisiológicas, el HGF es capaz de proteger contra dicho daño colestásico, ya que los animales “wild type”, cuya señalización asociada al HGF endógeno se encuentra activada, no presentaron tales alteraciones. También nuestro grupo ha reportado que los ratones MetKO presentan un cierto grado de daño colestásico *per se*, aún siendo alimentados con dietas normales. En efecto, el análisis transcriptómico global de estos ratones muestran afectaciones en rutas asociadas a la función hepatobiliar normal (Gomez-Quiroz, Seo et al. 2016). Todos estos resultados previos dan adecuado marco conceptual y sustentan el aporte que se hace en esta tesis en el campo de la terapéutica experimental al demostrar que dosis farmacológicas de HGF reforzarían su función basal anticolestásica, permitiendo contrarrestar el daño colestásico.

Finalmente, y como un aporte también relevante en este trabajo de investigación, se ha provisto evidencia experimental de que el efecto protector del HFG en colestasis se extiende a la función y estructura renal. En la colestasis inducida por ANIT, la función renal se vio seriamente afectada, con aparición de altos niveles de albúmina en orina y marcadas alteraciones de la función renal. Ambos factores son indicativos de insuficiencia renal asociada a alteraciones en el proceso de filtración glomerular y estructurales (Pinto-Sietsma et al. 2000), y el HGF contrarrestó totalmente estas alteraciones. La alteración renal en colestasis (nefropatía colémica) ha sido asociada al efecto tóxico directo que los niveles elevados de sales biliares en plasma tendrían sobre el riñón, dado su rol alternativo excretor, cuando las mismas son redireccionadas de hígado en colestasis (Krones, Pollheimer et al. 2018). El rinón normalmente reabsorbe prácticamente todas las sales biliares que han filtrado de plasma vía el transportador de ácidos biliares dependiente de sodio apical (ASBT). Sin embargo, sus altos niveles sistémicos en colestasis saturarían dicho sistema de reabsorción tubular proximal, de modo de que las sales biliares remanentes en el lúmen urinario causarían injuría oxidante a las células tubulares, y la posterior liberación de mediadores vasoactivos por las células dañadas produciendo vasoconstricción renal, con la consiguiente reducción de la tasa de filtración glomerular (Fickert, Krones et al. 2013). Sustentando esta posibilidad, el análisis histológico de los riñones mostró la presencia de varios cilindros tubulares en las muestras de los animales tratados con ANIT, lo que sugiere un daño en el epitelio tubular, esto aunado a una dilatación importante en los túbulos, aspecto similar al observado en la nefropatía colémica experimental inducida por ligadura del conducto biliar común por un periodo más largo (8 semanas) (Fickert,

Krones et al. 2013). La sobrecarga renal de bilirrubina conjugada también pudo haber contribuido a la disfunción renal, ya que es capaz de producir alteraciones en la membrana plasmática y la estructura mitocondrial de células tubulares renales a niveles compatibles con los alcanzados en nuestro modelo (Gollan et al. 1976). Los conjugados de bilirrubina pueden producir también inhibición de la actividad fosforilativa mitocondrial y, por ende, de la función celular por depleción de ATP (Ozawa et al. 1979). Dado el rol central que el estrés oxidante asociado a colestasis tendría en la patología renal, nuestros datos demostraron el control por parte del HGF del estrés oxidante que indujo el ANIT en este órgano serían claves para comprender sus mecanismos de protección renal. Efectos beneficiosos directos del HGF sobre el riñón como los postulados aquí son factibles, ya que su receptor c-Met es expresado en tejido renal (Pisters et al. 1997).

Tomados colectivamente, nuestros datos muestran claramente que el HGF es capaz de inducir protección hepática y renal en desórdenes colestásicos, a través de múltiples mecanismos asociados a su capacidad para contrarrestar la generación de estrés oxidante y modular la expresión de transportadores de manera de posibilitar vías alternativas de depuración.

## 9. Conclusiones

Los resultados derivados de este trabajo de tesis revelan numerosos aspectos novedosos de protección que posee el HGF como agente anti-colestásico, desplegando numerosos mecanimos de protección tanto a nivel hepático como renal

en el modelo de colestasis inducido por el ANIT. El mecanismo de protección que el HGF despliega involucra su capacidad para activar al factor de transcripción Nrf2, y su modo de acción es dual. Por un lado, HGF posee efectos antioxidantes directos e indirectos que restauran el balance redox, ya sea incrementando enzimas antioxidantes (por ej., catalasa, superóxido dismutasa) y por otro lado favoreciendo la depuración de especies potencialmente pro-oxidantes (por ej., sales biliares y el ANIT mismo). Para posibilitar esto último, regula mecanismos de fase III de destoxificación, incrementando la expresión de transportadores hepatocelulares de extrusión basolateral y reprimiendo los de excreción canalicular, lo cual favorece la vía renal de depuración tras el redireccionamiento a circulación sistémica. Este último mecanismo anticolestásico particular le confiere al HGF una alta potencialidad para el tratamiento de enfermedades colestásicas crónicas obstructivas que no cuentan con recursos terapéuticos satisfactorios en la actualidad, como por ejemplo la colangitis esclerosante primaria, de la cual la colestasis por ANIT es un buen modelo. Los efectos protectores renales que adicionalmente despliega el HGF también contribuyen a reforzar su potencialidad terapéutica, dada la alta incidencia de lesión renal aguda asociada al fenómeno colestásico (Fickert, Krones et al. 2013). Esto ha puesto de manifiesto la necesidad urgente de establecer alternativas terapéuticas específicas, y el HGF, por sus mecanismos pleiotrópicos de protección, podría ser una de ellas. Sin duda, los avances que se están realizando en el campo de la terapéutica para favorecer tales procesos regenerativos y controlar simultáneamente efectos colaterales no deseables repercutirán en la oportunidad de su efectiva utilización como

agente con doble propiedades beneficiosas, tanto anticolestásicas como regenerativas.

Los resultados del presente trabajo de tesis han sido publicados en *Biochemical Pharmacolology* (Salas-Silva et al. 2020), revista arbitrada, indizada y de difusión internacional. El artículo se agrega al final del documento en anexos, junto con otras publicaciones producto de la investigación doctoral.

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## 11. Anexos

### *11.1 Daños provocados por una administración sostenida de ANIT.*

Durante la realización del presente trabajo, se realizaron experimentos preliminares del efecto que tiene el ANIT en ratones tratados con este colestásico de manera sostenida, para lo cual se realizó el siguiente diseño experimental de administración de dosis múltiples aplicadas a diferentes tiempos de administración:

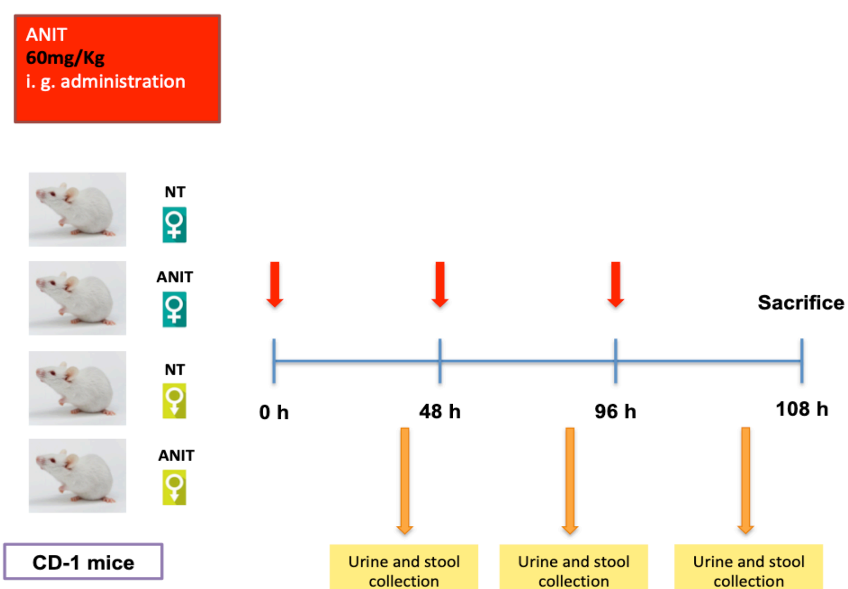


***Figura A1. Diseño experimental de aplicación de dosis múltiples de ANIT a diferentes tiempos de administración***

### Diseño Experimental

16 ratones CD-1, 8 machos y 8 hembras, se dividieron aleatoriamente en 2 grupos en jaulas metabólicas, con alimento estándar y agua *ad libitum*. Los ratones se trataron o no con 3 dosis de ANIT de 60 mg/kg, i.g., cada 48 h. La recolección de orina se realizó la noche antes de cada administración de ANIT (Figura A1). Ciento ocho horas

después de la primera dosis de ANIT, los animales fueron sacrificados bajo anestesia con isoflurano (2% de isoflurano y 2 l/min de oxígeno). Se obtuvieron muestras de sangre, hígado, riñón, páncreas e intestino.

**El ANIT, administrado de manera prolongada, induce daño multiorgánico**

En la Figura A2 la inspección macroscópica de los órganos reveló hepatomegalia, la cual se corroboró en la Figura A3A, ligera hipertrófia y cambio en la coloración de la vesícula biliar en los animales ANIT. Resulta interesante que, en los grupos tratados con el colestásico, los hígados de los machos, a diferencia de las hembras, presentaron lesiones que podrían ser zonas necróticas. No se observaron cambios macroscópicos aparentes en el tejido renal. El páncreas de los animales tratados con ANIT mostró un ligero creciemineto en ambos sexos, lo que podría sugerir compromiso de este órgano debido al proceso colestásico, hipótesis a ser confirmada con un análisis cuantitativo. Por último, llamativamente, el intestino de los animales tratados con ANIT presentó una disminución en el grosor de la pared intestinal que se apreció a simple vista, ya sea en hembras como en machos, lo cual sugiere que el intestino tambíen está siendo afectado por efecto del colestásico. El efecto de ANIT sobre el intestino resultó altamente interesante al compararlo con el obtenido en el tratamiendo agudo con una única dosis de ANIT, donde los animales tratados con esta droga presentaron una disminución de heces fecales, lo cual podría estar realcionado con algún desorden a nivel intestinal que se vuelve a manifestar en estos animales luego del tratamiento prolongado.

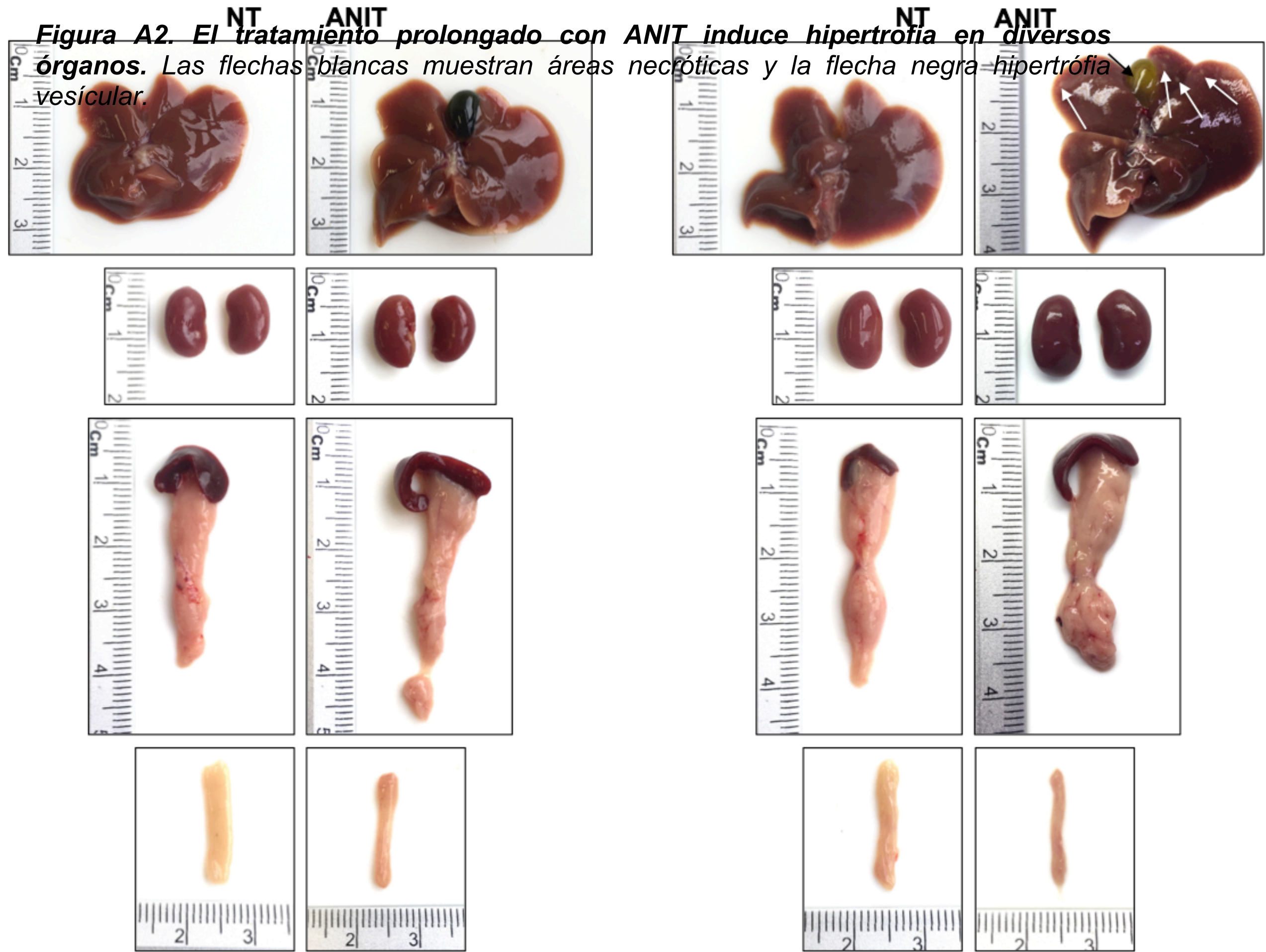


***Figura A2. El tratamiento prolongado con ANIT induce hipertrofia en diversos órganos.*** *Las flechas blancas muestran áreas necróticas y la flecha negra hipertrófia vesícular.*

**Las pruebas de funcionalidad hepática incrementaron con el tratamiento prolongado con ANIT**

También evaluamos el perfil bioquímico de los animales sometidos a un tratamiento prolongado, con múltiples dosis con ANIT. Los niveles de AST (Figura A3B), ALT (Figura A3C), ALP (FiguraA3D) y las sales biliares en suero (Figura A3E) incrementaron significativamente en los ratones tratados con ANIT de manera independiente del sexo en compración con los grupos control, confirmando la presencia de daño en estos animales, aunque a nivel macroscópico los hígados de las hembras no presentaban lesiones en forma de nódulos.

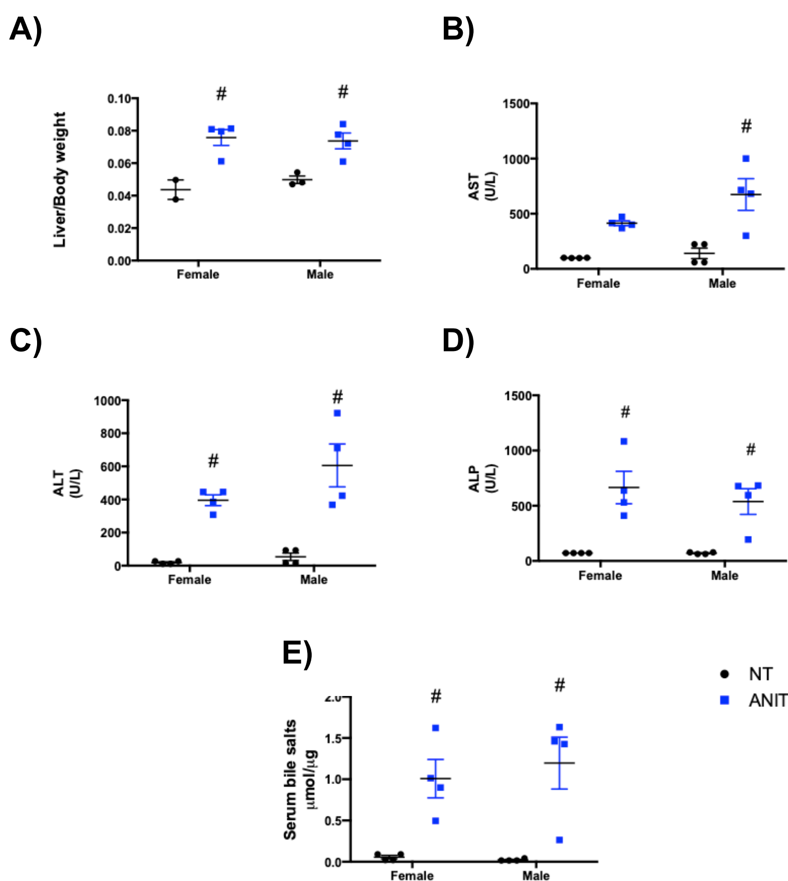


***Figura A3. Parámetros morfométricos y bioquímicos en machos y hembras luego de la administración prolongada de ANIT.*** *A) Cociente hígado/peso del ratón. B) Niveles séricos de AST, C) Niveles séricos de ALT. D) Niveles séricos de ALP. E) Contenido de sales biliares en suero.* $^{\#}p<0.01$ *vs NT.*

**La posible disfunción renal inducida por ANIT es dependiente del tiempo y del sexo**

Por último, la inspección de la primera recolección de orina mostró un cambio en la coloración urinaria, como se reportó anteriormente en el modelo agudo, siendo más evidente este cambio en la segunda recolección (Figura A4A) y casi imperceptible en la tercera recolección, lo cual podría deberse a un mecanismo de adaptación al ANIT. Se evaluó la presencia de HSP72 en la orina de estos animales, y observamos un incremento significativo en este marcador en la orina de la segunda recolección para ambos sexos (Figura A4B) coincidiendo con el cambio de coloración de la orina. Con base en esto, podríamos inferir que el pico máximo de daño renal se encuentra antes de las 84 h, con 2 dosis de ANIT previamente administradas. Una observación interesante es que, en la primera recolección de orina, es decir antes de las primeras 48 h de exposición al ANIT, los ratones machos presentaron un incremento en esta proteína, tal como se reportó anteriormente. Sin embargo, a este mismo tiempo, las hembras no presentaron daño aparente. Por lo anterior, podríamos inferir que existe una diferencia en los mecanismos de daño y reparación dependiente del sexo, siendo los machos más suceptibles al daño por ANIT que las hembras.

Los anteriores resultados son parte de un nuevo modelo que estamos proponiendo en el grupo de investigación para evaluar si HGF es capaz de proteger al ratón a nivel multiorgánico ante una agresión sostenida de ANIT, y si el sexo es un determinante para el establecimiento y progresión del daño.

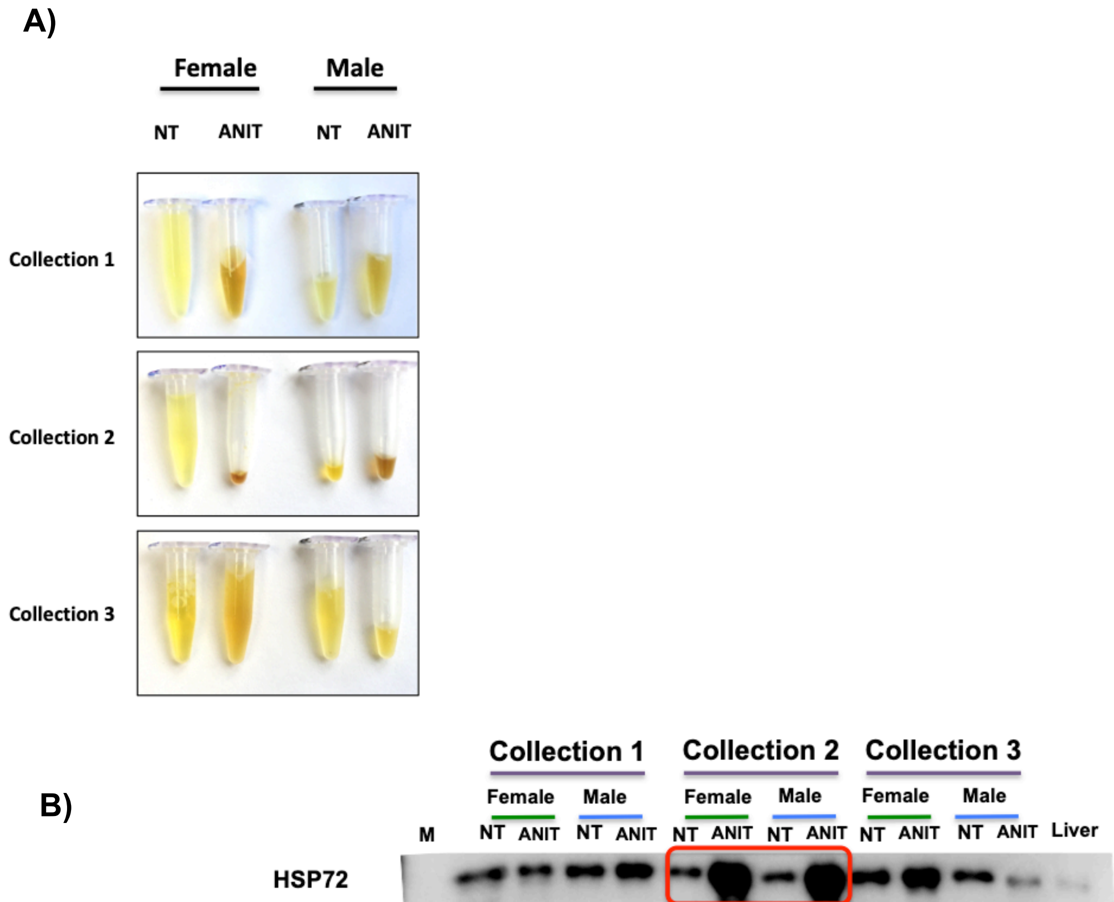


***Figura A4. Análisis urinario del efecto de ANIT administrado en forma prolongada.***
*A) Inspección de la coloración de la orina. B) Western blot de HSP72.*

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# HGF induces protective effects in α-naphthylisothiocyanate-induced intrahepatic cholestasis by counteracting oxidative stress

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A R T I C L E I N F O

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A B S T R A C T

Cholestasis is a clinical syndrome common to a large number of hepatopathies, in which either bile production or its transit through the biliary tract is impaired due to functional or obstructive causes; the consequent intracellular retention of toxic biliary constituents generates parenchyma damage, largely via oxidative stress-mediated mechanisms. Hepatocyte growth factor (HGF) and its receptor c-Met represent one of the main systems for liver repair damage and defense against hepatotoxic factors, leading to an antioxidant and repair response. In this study, we evaluated the capability of HGF to counteract the damage caused by the model cholestatic agent, α-naphthyl isothiocyanate (ANIT). HGF had clear anti-cholestatic effects, as apparent from the improvement in both bile flow and liver function test. Histology examination revealed a significant reduction of injured areas. HGF also preserved the tight-junctional structure. These anticholestatic effects were associated with the induction of basolateral efflux ABC transporters, which facilitates extrusion of toxic biliary compounds and its further alternative depuration via urine. The biliary epithelium seems to have been also preserved, as suggested by normalization in serum GGT levels, CFTR expression and cholangyocyte primary cilium structure our results clearly show for the first time that HGF protects the liver from a cholestatic injury.

## 1. Introduction

Cholestasis is a clinical condition common to a large number of liver diseases, originated from impairment in bile production or the bile transit through the biliary tree, thus resulting in a decreased amount of bile reaching the duodenum. The cause of this alteration can be a primary impairment in the capacity of hepatocytes to generate bile (hepatocellular or metabolic cholestasis) or a mechanical obstruction of the bile transit (obstructive cholestasis) [1].

Efflux of solutes by ATP-binding cassette (ABC) transporters is the main determining factor in the general capacity of the liver tissue to secrete bile and to detoxify it from endo and xenobiotics [2]. Bile flow generation is driven mainly by the biliary excretion of bile salts and glutathione, which account for the so called bile salt-dependent and bile salt-independent fraction of bile flow, respectively [2]. The canalicular transporter bile salt export pump (Bsep) is the main bile salt transporter, whereas the human multidrug resistance-associated protein 2 (Mrp2) is the main transporter of glutathione into bile, and also of bilirubin glucuronates and a wide range of glucuronide and glutathione conjugated with endo- and xenobiotics. In addition, other members of this transport family, such as Mrp3 and Mrp4, are located in the basolateral membrane, and mediated the extrusion of numerous bile salts

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and several glutathione-, glucuronic acid-, and sulfate-conjugates [1]. Cholestatic hepatocytes attempt to limit intracellular accumulation of bile salts and other toxic biliary compounds by inducing these basolateral hepatocellular export pumps, thus allowing their urinary excretion. Also, adaptive changes in the proximal renal tubule occurs, which collectively facilitates their renal elimination at the expense of increasing the bile salt burden for the renal tubular system [3].

α-Naphthyl isothiocyanate (ANIT) is a hepatotoxicant that has been used primarily as a tool to study the pathogenesis of chemical-induced cholestasis, particularly drug-induced cholestasis. Its mechanisms of cholestasis involves impairment of bile salt transporter expression, disruption of hepatic tight junction and, lately, biliary obstruction due to desquamation of the epithelial cells of the bile duct [4]. ANIT also causes death of hepatocytes and cholangiocytes by necrosis and apoptosis, both by direct toxic action and, indirectly, through the generation of an intense inflammation associated with the infiltration of neutrophils around the hepatocytes and bile ducts; this inflammatory state leads to the production of reactive oxygen species (ROS), thus inducing oxidative stress which can also cause death of hepatocytes and cholangiocytes, with the subsequent formation of obstructive cellular debris [5]. Many of these alterations are similar to that occurring in obstructive cholangiopathies such as primary sclerosing cholangitis[6] and this is why ANIT-induced cholestasis has been used as an experimental model to mimic this disease [7].

Hepatocyte growth factor (HGF) and its receptor, cellular-Mesenchyme Epithelium Transition factor (c-Met), represent one of the main system for liver repair damage and defense against different hepatotoxic factors, as extensively proved by our group [8–11]. In addition, HGF is required for repairing process in the kidneys [12], lung [13], and pancreas [14], among other organs. It also affords protective effects on epithelial and non-epithelial organs, such as the heart and the brain [15]. This points c-Met signaling as a critical factor in protecting multiple organs against over-generation of ROS [9,16]. That is why the role of HGF/c-Met has been characterized in the regulation of the cellular redox state and the oxidative stress in the liver [8]. HGF/c-Met regulates the activation of key transcription factors that drive the expression of antioxidant and survival genes, such as nuclear factor kappa B (NF-κB) or the nuclear factor-erythroid 2-related factor 2 (Nrf2), leading to cell survival response under oxidative insults [16].

Nrf2 regulates the expression of a broad range of antioxidant and cytoprotective genes, such as NADPH and γ-glutamylcysteine synthetase, hepatocellular transporters, such as Mrp2, Mrp3, Mrp4, Bsep, OATP, OSTα, and phase I metabolizing enzymes, such as Cyp7a1 and Cyp8b1 [16,17].

Currently, there is no effective treatment for cholestatic diseases due to little knowledge of their pathogenesis. This is why cholestatic diseases are the main cause of liver-associated disease, leading to cirrhosis and progressive liver failure that requires liver transplant for the patient to survive [18]. Although, some limited approaches have been reported regarding the protective effect of HGF in ANIT-induced cholestasis in the rat [19], the mechanism of action remained unclear. For this reason, the aim of this work was to characterize the anti-cholestatic effect of HGF in a mouse model of chemical cholestasis induced by ANIT.

## 2. Materials and methods

### *2.1. Animal models*

Forty 10-weeks-old CD1 male mice were obtained from the Universidad Autonoma Metropolitana Iztapalapa (UAM-I) animal facility and were maintained in pathogen-free conditions with controlled temperature and humidity on a 12 h light-dark cycle in the same facility. The experimental protocols used were approved and carried out in accordance with the UAM-I experimental animal guidelines and NIH Guide for the Care and Use of Laboratory Animals.

### *2.2. Experimental design*

CD1 mice, placed in metabolic cages, were treated or not with ANIT (60 mg/kg, intragastric, Sigma Aldrich, USA, #N4525 diluted in soy oil) for 48 h. After 24 h of ANIT treatment, HGF (10 μg/kg, intravenous, Peprotech, Rocky Hill, NJ, USA, #100–39) was administrated. Forty-eight hours after ANIT treatment, animals were euthanized under isoflurane anesthesia (2% isoflurane and 2 l/min oxygen). Blood, bile, and liver tissue were obtained. Food and water were given *ad libitum*.

### *2.3. Serum and urine biochemical determinations*

Blood samples were obtained from the orbital venous plexus under Anesthesia (2% isoflurane and 2 l/min oxygen). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (GGT) were determined, by using automated method (SpotChem EZ, ARKRAY, USA).

### *2.4. Bile salt and bilirubin determination*

Serum and tissue bile salts content were assayed by using Total Bile Acid Assay (DIAZYME Laboratories, CA, USA) and bilirubin through Jendrassik-Grof FS kit (DiaSys Inc, Canada), following the manufacturer's instructions.

### *2.5. Hematoxylin-Eosin staining (H-E)*

Histology was performed by routine H-E staining, as previously reported [13].

### *2.6. Bile flow studies*

Bile was collected under isoflurane anesthesia. The common bile duct was cannulated with a polyethylene tubing (PE10). Bile was collected for 30 min, and bile flow was determined gravimetrically, by assuming a density of 1 g/ml, as previously reported [20].

### *2.7. Bile salts elimination in bile*

Total bile salts were determined by the 3α-hydroxysteroid dehydrogenase procedure [21].

### *2.8. ROS in situ determination*

After mice euthanasia, fresh liver tissue was rapidly obtained and sectioned, frozen in liquid nitrogen, and embedded in optimum cutting temperature reagent (OCT, Sakura Finetec, Torrance, CA, USA). Immediately, 8-μm frozen sections were obtained in a cryostat (Leica CM-3050S) at −20 °C, and the slides were then incubated for 15 min with dihydroethidium (DHE, 5 μM #D11347, Invitrogen, CA, USA), in the dark and at room temperature, for determination of superoxide radical detecting ethidium fluorescence. Samples were observed using a confocal microscope (Carl Zeiss 780 LSM-NLO), at excitation and emission wavelengths of 485 and 570 nm, respectively, as we previously reported [8].

### *2.9. Lipid peroxidation*

Lipid peroxidation was assayed by the production of thiobarbituric acid reactive substances (TBARS), using a spectrophotometric method [22].

### *2.10. Western blot*

Western blotting was carried out following the protocol previously reported [23]. Briefly, total proteins were isolated from liver

homogenate with T-PER (Pierce, Invitrogen, CA, USA) extraction reagents, supplemented with protease and phosphatase cocktail inhibitors (Roche Inc, Switzerland). One hundred µg of total protein was separated on precast 4–20% gels (Invitrogen Inc, CA, USA), transferred to polyvinylidene difluoride membrane (PVDF, Invitrogen, CA, USA), and probed with anti-CFTR antibody (Abcam #2784, dilution 1:1000, target protein size 168 KDa) and ZO-1 antibody (Invitrogen #617300, dilution 1:1000, target protein size 195 KDa). Membranes were incubated with anti-mouse/rabbit horseradish peroxidase-conjugated secondary antibody. Immunoreactive band was identified with ECL-Plus Western blotting detection reagents (GE Healthcare). Equal loading was demonstrated by probing the same membrane with actin antibody (Sigma-Aldrich #A2060, dilution 1:10000, target protein size 42 KDa).

### 2.11. Gene expression determination by real-time RT-PCR

Quantification of mRNA levels for five selected HGF/Met target genes was performed. Total RNA was obtained using TRIzol RNA isolation reagent (# 15596018, Thermo Fisher Scientific, USA) following manufacturer's instruction. After DNase digestion with a DNA-free kit (Ambion Inc, Thermo Fisher Scientific, USA), 1 µg of total RNA was reverse transcribed in 20 µl reaction volume with a SuperScript (#170-8891 Invitrogen, CA, USA) first-strand synthesis kit, according to the manufacturer's instructions. Real-time quantitative PCR analysis was carried out with a CFX96 Touch (Bio-Rad) thermal cycler in a 96-well reaction plate. The 10-µl PCR reaction mix contained 5 µl 2X SYBR Green PCR Master Mix (#172-5016 Bio-Rad Laboratories, USA), 200 nM of each primer, and 1 µl cDNA template. Reactions were incubated for 10 min at 95 °C, followed by 40 cycles of 30 sec at 95 °C and 60 sec at specific primer temperature. Melting analysis of the PCR products was also conducted to validate the amplification of the specific product. The expression level of the mouse ribosomal protein S18 (rps18) was used as an internal reference. Relative gene expression levels were calculated with the $2^{-\Delta\Delta ct}$ method. Primer sequences used in the study are listed in Table 1.

### 2.12. Canalicular width studies by immunofluorescence and image analysis

To assess the structural status of the paracellular tight junctions that seals the bile canaliculus, immunolocalization of the tight junction-associated protein ZO-1 was studied by confocal microscopy (Zeiss Pascal LSM 5, Carl Zeiss, Germany). For this purpose, a liver lobe was excised, and frozen immediately in isopentane precooled in liquid nitrogen, and stored at −80 °C. The, liver samples were sectioned and fixed, and ZO-1 was labeled with polyclonal antibody (#617300, Invitrogen, CA, USA). Immunostaining was completed by treatment of the preparations with appropriate Cy3-conjugated mouse anti-IgGs (Jackson Immuno Research Laboratory, Inc. West Grove PA). Analysis of images was carried out by using the Image J 1.34 m software (NIH). The average canalicular width of each group was assessed as a first approach to correct for distortions in fluorescence intensity induced by changes in canalicular volume, as a result of the treatments. Assessment of the percentage of 'double rail' canalicular, and canalicular width, in confocal images was also performed. To assess the proportion of canalicular double rail structures, the presence of both single and double canalicular rails was quantified, and then the number of double rail structures was divided by the total (single and double rail) canalicular structures. Alternatively, canalicular structures were addressed in liver tissue from c-MetKO mice using rhodamine-phalloidin reagent (#235138, Abcam) in combination with ZO-1 antibody IF. Liver tissue from c-MetKO mice was generously donated by professor Gomez-Quiroz.

### 2.13. Bile duct structure by scanning electron microscopy (SEM)

SEM Micrographs were obtained with a scanning electron microscope (JEOL JSM-5900 LV). All samples were fixed in 3% glutaraldehyde for 48 h and incubated with osmium tetraoxide for 2 h and dehydrated in 30–100% ethanol. After drying using the critical point dryer, the samples were coated with gold. The SEM was operated at 15 kV accelerating voltage [24].

### 2.14. Statistical analysis

Data are presented as mean ± SEM for at least 4 different animals; each experiment was carried out in triplicate. For the comparison of means of different groups, an analysis of variance (ANOVA) was used, followed by multiple comparisons by Tukey test. The Graph Pad Prism version 8 software for MacOS was used. The level of significance considered was $p \leq 0.05$.

## 3. Results

### 3.1. ANIT induces time-dependent hepatobiliary damage

In order to confirm the ANIT-induced hepatobiliary damage as reported [25,26], we first evaluated the changes with time of the biochemical liver function test assayed in mice treated with ANIT (60 mg/Kg, intragastric). Fig. 1A shows a remarkably increment in serum activity of AST, and indicator of hepatocellular necrosis, since 12 h of treatment onwards, maintaining this value up to 48 h. Total and direct serum bilirubin levels were elevated later, with increments reaching statistical significance at 48 h of ANIT administration, although a tendency towards increased levels can be seen at 36 h of ANIT exposure. The levels of intracellular bile salt retention was evaluated by assessing serum levels of ALP, a plasma membrane-bound hepatocellular enzyme that is induced and released into blood by the detergent bile salts accumulated in cholestasis [27]. Fig. 1 shows an significant increment of the enzyme since 36 h onwards, reaching a maximal value at 48 h; this correlated with the serum bile salt levels, which increase at 36 h, although they modestly decreased at 48 h, perhaps reflecting induction of renal transporters to improve alternative urinary bile salt excretion [3].

Gross liver examination exhibited macroscopic liver lesions (white arrows) with hepatomegaly (Fig. 1B), consistent with an increment in the liver to body weight ratio (Fig. 1C). The organ also revealed gallbladder swelling and dark coloring (Fig. 1B, black arrow).

The liver histology showed prominent necrotic areas (asterisk) and inflammatory infiltration (black arrows and inset), as compared with livers from not treated animals (Fig. 1D). These data clearly show that ANIT induces hepatobiliary damage, although the evident injury is detected from 36 h onwards.

Since a typical cholestatic phenotype is observed at 48 h, in the

**Table 1**
PCR Primers used in the study.

| Protein name | Acronym | Gene | Foward | Reverse |
|---|---|---|---|---|
| Bile salt efflux pump | Bsep | *Abcb11* | GGCTTGCTACAGATGCTTCC | GCCAAAAAGGGGAAGAAGAC |
| Multidrug resistance-associated protein 2 | Mrp2 | *Abcc2* | CTGAGTGCTTGGACCAGTGA | CCCACAGTCACCATCCTCTT |
| Multidrug resistance-associated protein 3 | Mrp3 | *Abcc3* | CCCTGCTCCTGTCTTCTTTG | GCTGAGAGGATCTTGGAACG |
| Multidrug resistance-associated portein 4 | Mrp4 | *Abcc4* | CATACCATTGGTTCCGCTCT | GTCAGGAGCTGTTTGATGCA |
| Ribosomal protein s18 | s18 | *rps18* | TGTGGTGTTGAGGAAAGCAG | TCCCATCCTTCACATCCTTC |

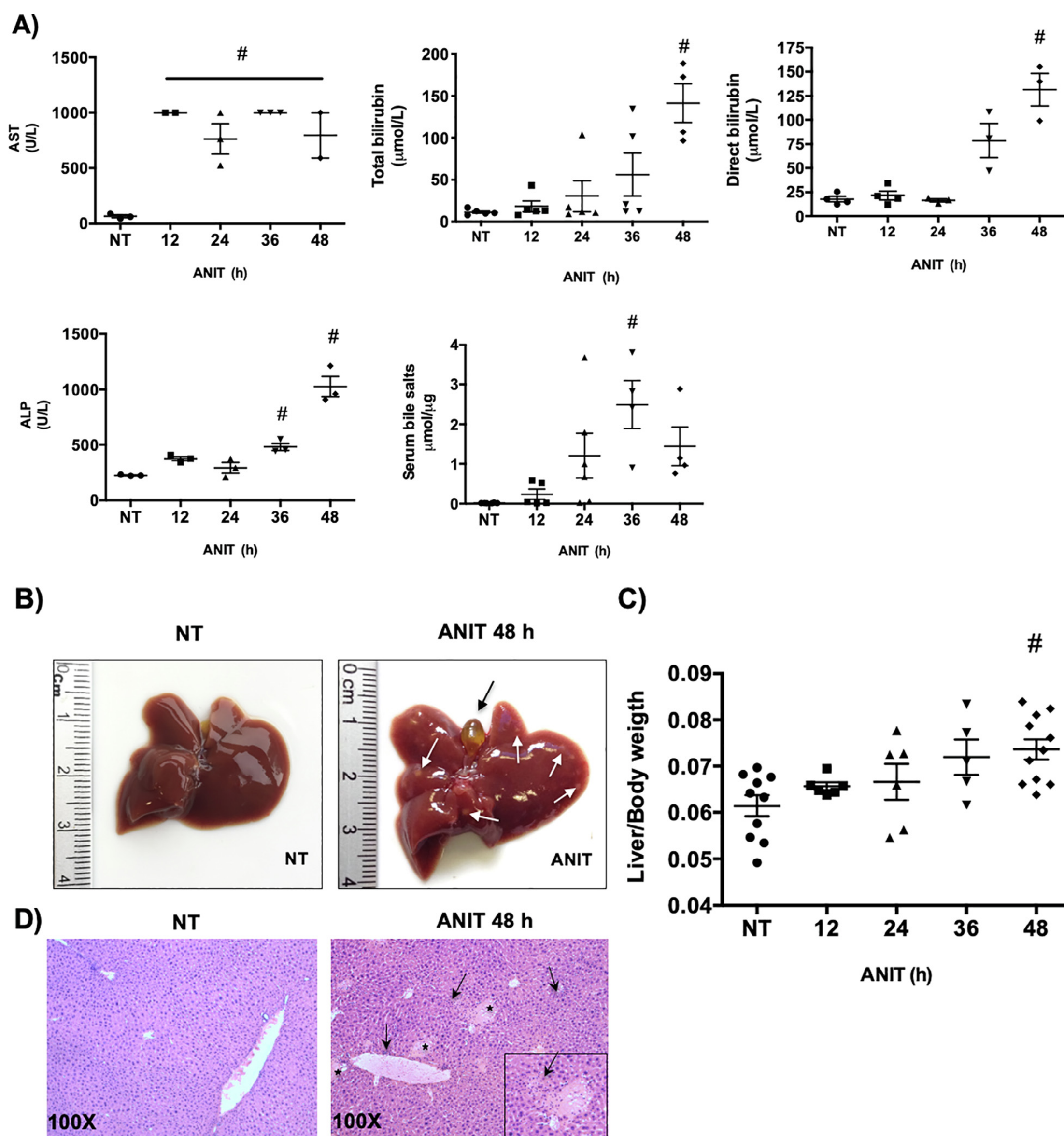


**Fig. 1.** ANIT induced liver damage determined by liver functional tests. A) Aspartate aminotransferase (AST) activity, Total bilirubin, Direct bilirubin concentration, Alkaline phosphatase (ALP) activity and Serum bile salt content. B) Macroscopic liver inspection of ANIT treatment (white and black arrows show nodulary and gallbladder swelling and dark coloring respectively). C) Liver-to-body weight ratio. D) Histology appearance by H&E staining (black stars indicate areas with necrosis, and arrows show inflammatory infiltrate). The images are representative of at least 3 experimental animals and are shown at 100× the original magnification. Each group represents the median ± SEM in at least four different mice, $^{\#}p < 0.01$ vs NT.

remaining experiments we decided to explore the effects of HGF at this time.

### 3.2. HGF has anticholestatic properties

As we had previously reported, HGF induces hepatoprotective properties in the liver [8,28]. To ascertain whether this protection could be displayed under a cholestatic condition induced by ANIT, we treated animals with this chemical and we evaluated HGF capability to normalize a number of biochemical liver function tests in mice (Fig. 2A). HGF treatment significantly decreased AST, ALT, ALP, and GGT serum levels (27.6, 7.4, 2.6 and 2.8-fold, respectively). Even more, we observed a significant drop in total (5.4-fold) and direct bilirubin (10.9-fold) in the co-treated group, as compared with mice treated only with ANIT. Similarly, the treatment with HGF fully normalized bile salt levels in both serum and tissue, by improving bile salt levels 19.3-fold and 1.7-fold, respectively.

These data were in agreement with macroscopic inspection of the liver (Fig. 2B). Animals treated with ANIT + HGF presented a significant decrease in tissue lesions, nodularity (white arrows), gallbladder swelling and coloring, and the hepatomegaly decreased to normal values, as shown in Fig. 2C.

The H-E staining showed preservation of the tissue integrity in samples from co-treated animals, as compared to those treated with

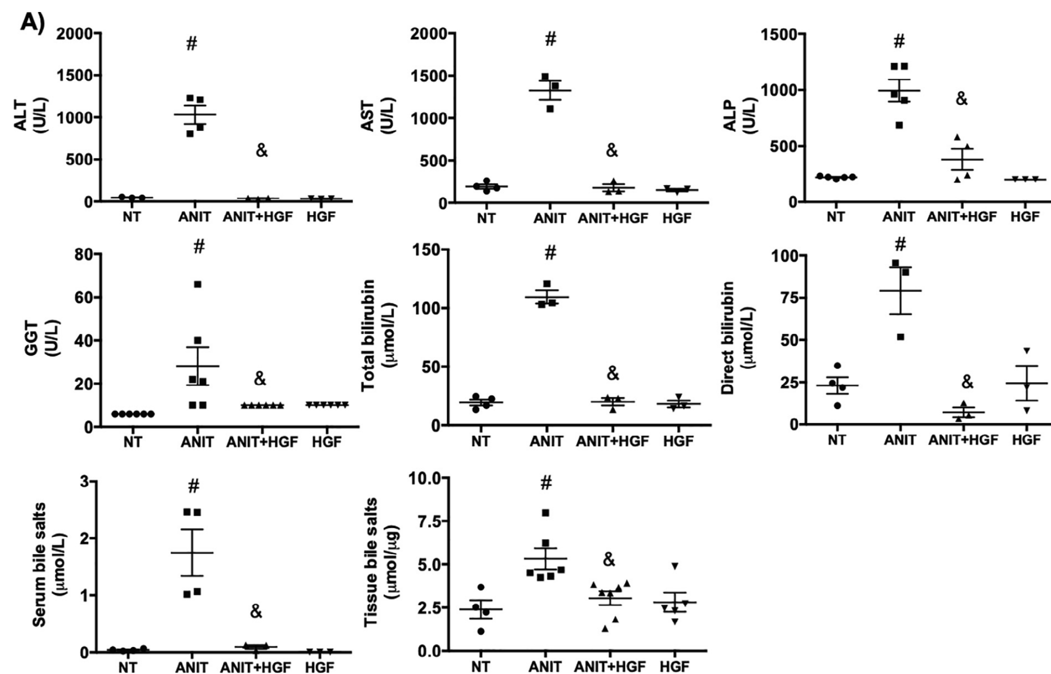


**Fig. 2.** HGF protects against the cholestatic damage induced by ANIT. A) Alanine aminotransferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), γ-glutamil transpeptidase (GGT) activity, total and direct bilirubin concentration, and the serum and tissue levels of bile salts. B) Macroscopic liver inspection of treated groups (white and black arrows show nodulary). C) Liver-to-body weight ratio. D) Histology appearance by H&E staining (black stars indicate areas with necrosis, and arrows show inflammatory infiltrate). The images are representative of at least 4 experimental animals and are shown at 50× and 100× the original magnification. E) Bile flow. Each group represent the median ± SEM in at least four different mice, $^{\#}p < 0.01$ vs NT, $^{\&}p < 0.01$ vs ANIT. F) Bile salts biliary excretion. Each bar represents the median ± SEM in at least four different mice.

ANIT alone; the necrotic areas (asterisk) were significantly decreased by HGF, and inflammatory infiltration was not observed (Fig. 2D).

### 3.3. HGF restores ANIT-induced bile flow impairment

Bile flow decreased approximately 1.7-fold in ANIT group as compared to the non-treated (NT) group. However, under HGF co-treatment, bile flow was restored to normal values (Fig. 2E), together with a tendency towards increase bile salt output, which did not reach statistical significance (Fig. 2F); this suggests preferential exportation of accumulated bile salts via urine rather than via the biliary route. HGF induced neither choleresis nor increase in bile salt output *per se*.

### 3.4. HGF diminishes ANIT-induced oxidative stress

To ascertain whether HGF improves the cholestasis by normalizing redox homeostasis, we evaluated the levels of superoxide radical ($O_2^{\cdot-}$) in fresh liver sections in all groups. The confocal images showed that ANIT treatment increased significantly $O_2^-$ cellular content (20-fold) versus the NT group. However, in the co-treated group, this ROS exacerbated levels considerably diminished (Fig. 3A). This was in agreement with the lipid peroxidation data, which were fully normalized by HGF (Fig. 3B), thus confirming that HGF decreases the oxidative damage induced by ANIT exposition.

### 3.5. HGF preserves the tight-junctional structure disturbed by ANIT

To explore further the possible mechanisms underlying the anticholestatic effects of HGF, and since integrity of tight-junctional complexes that seal the bile canaliculus are required to maintain the bile-to-blood gradients that drive bile formation [29], we analyzed the effect of HGF on the disorganization of these structures, by studying the localization of the tight-junctional protein zonula occludens-1 (ZO-1). To analyze this disorganization at the structural level, we addressed the status of the "double rail" or "train track" [30] canalicular structure by ZO1 immunofluorescence [31]. As shown in Fig. 4A, under normal conditions the bile canaliculus is defined as an intercellular space formed by the apposition of the edges of gutter-like hemicanals delimited by tight junctions, on the facing surfaces of adjacent hepatocytes [32], and this could be identified by a "double rail"-like structure [31], (Fig. 4A, yellow arrow and inset). ANIT treatment induced a decrease in the number of canalicular "double rail" structures (Fig. 4B). Actually, this group predominantly showed a "single rail" feature (white arrow), with a spiral-like structure. HGF cotreatment efficiently preserved the double rail structure similar to that in the NT group (Fig. 4B). Remarkably, HGF alone treatment substantially increased the canalicular width (green arrow) as compared with that of the NT group (1.4-fold) and normalized the decrease in this parameter induced by ANIT (Fig. 4C).

Interestingly, liver tissue from c-MetKO animals [9], (Fig. 4D), presented same abnormalities in the canalicular structure as those observed in both ANIT-treated mice and in the β-catenin KO mouse [30], with no changes in ZO-1 content (Fig. 4E).

These data suggest the HGF involvement in the organization of tight-junctional structures, which are required for normal bile secretion.

### 3.6. HGF normalize impairment of cystic fibrosis transmembrane conductance regulator (CFTR) induced by ANIT

A common feature in cholangiopathies is the defective ductular secretion of a bicarbonate-rich fluid secretion, which is driven by the

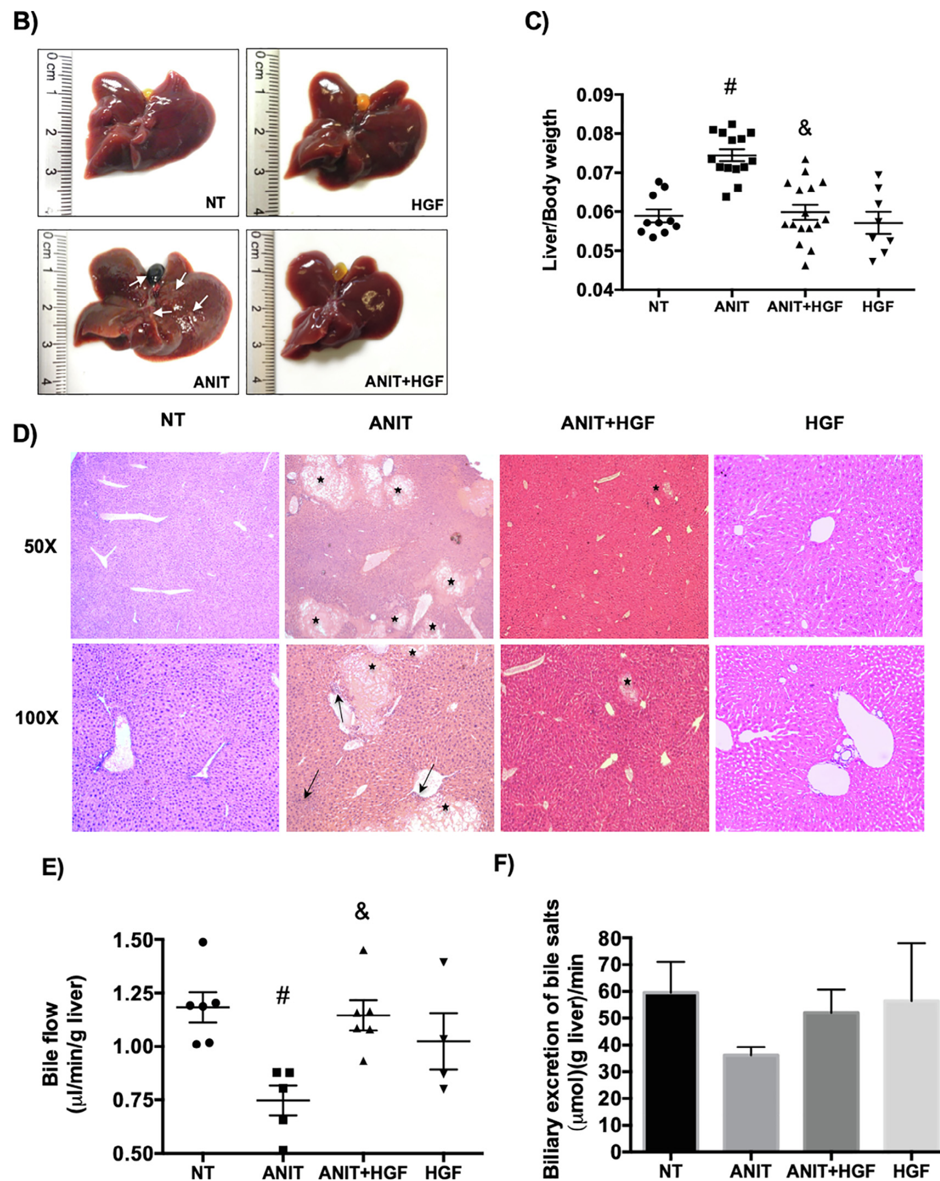


**Fig. 2.** (*continued*)

ATP-dependent chloride channel, CFTR [33]. Since this ductular bicarbonate-rich secretion has protective effects against bile salt -induced bile duct damage in cholestasis (the so called "bicarbonate umbrella") [34], CFTR function is considered to be a critical factor in protecting the biliary epithelium in cholestatic cholangiopathies. We therefore decided to explore the status of this apical membrane protein in our model. As shown in Fig. 4F, ANIT induced a significant decrease in CFTR expression, and co-treatment with HGF restored this parameter to basal values. Furthermore, HGF had *per se* a stimulatory effect on CFTR expression.

### 3.7. HGF protects the cholangiocellular cilium from ANIT damage

Cholangiocyte primary cilia are important for normal ductular bile secretion, and defects in its structure and/or function lead to altered ductular fluid secretion [35]. Therefore, we explore the damage caused by ANIT in cholangiocytes, particularly in the primary cilia, at 48 h of exposure. We assessed morphology by scanning electron microscopy (SEM). The primary cilia were shorter and virtually absent, and HGF cotreatment restored the normal cilium morphology (Fig. 5).

### 3.8. HGF induces basolateral efflux ABC transporters expression

Alterations in ABC transporters play an important role in the cholestatic process [1]. For this reason, we evaluated the genetic expression of the *Abcc2*, *Abcb11*, *Abcc3*, *and Abcc4* genes. *Abcb11*/Bsep is a canalicular transporter that exports bile salts into bile, whereas *Abcc2*/Mrp2 excrete glutathione and endo- and xenobiotics conjugated with

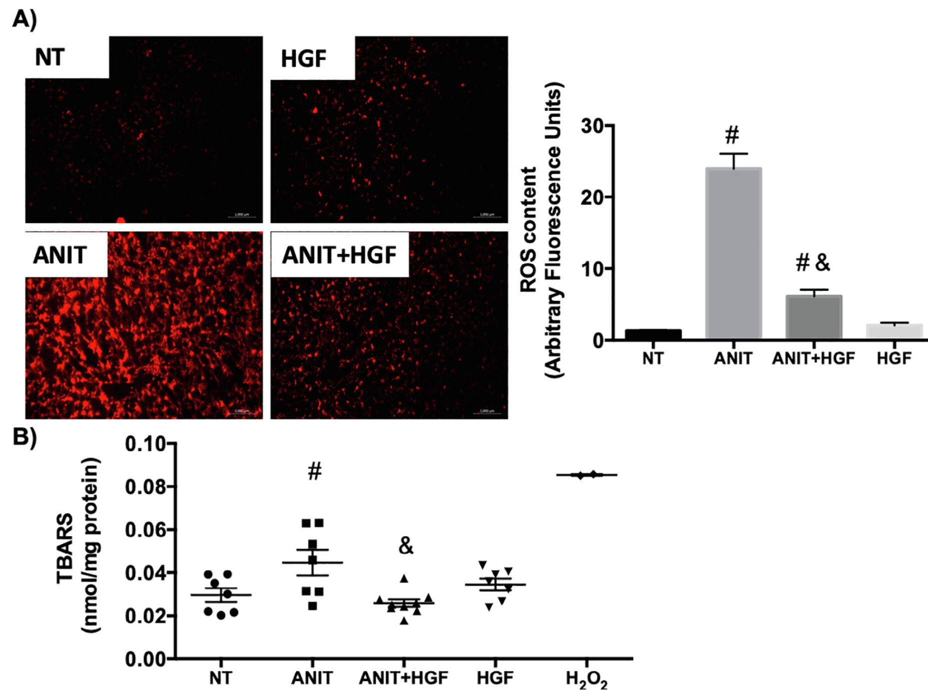


**Fig. 3.** HGF counteract the alterations in redox homeostasis induced by ANIT. A) Representative images obtained by confocal microscopy (original magnification X100) of superoxide content in liver tissue, as visualized in red with the superoxide probe dihydroethidium (left panel), and the corresponding fluorescence quantification of superoxide tissular levels by densitometric analysis (right panel). B) Lipid peroxidation determination assayed by thiobarbituric acid reactive substances (TBARS). Each group represents median ± SEM in at least five different mice. $^{\#}p < 0.01$ vs NT, $^{\&}p < 0.01$ vs ANIT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

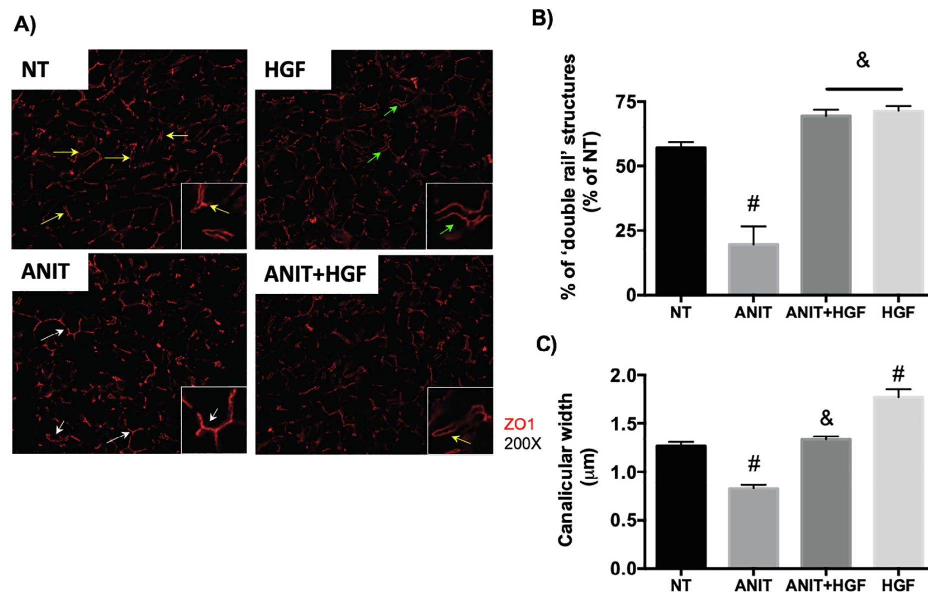


**Fig. 4.** HGF counteract the structural alterations in tight junctions induced by ANIT. A) Immunofluorescence determined by confocal microscopy of ZO-1 (yellow, white and green arrows show double rail, mono rail, and wider double rail respectively), Original magnification 200×. B) Quantification of the proportion of double rail canalicular structure. C) Canalicular width quantification. D) Representative confocal image composition of immunofluorescences of canalicular structure in liver tissue from untreated wild-type (WT) and c-MetKO. Rhodamine-phalloidin (red), ZO-1 (green). DAPI was used for nuclei identification (blue). E) ZO-1 content addressed by Western blot in liver tissue from c-MetKO and WT mice. The images are representative of at least 4 experimental animals. F) Western blot of CFTR and densitometric analysis. The images are representative of at least 3 experimental animals. Each bar represents median ± SEM, $^{\#}p < 0.01$ vs NT, $^{\&}p < 0.01$ vs ANIT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

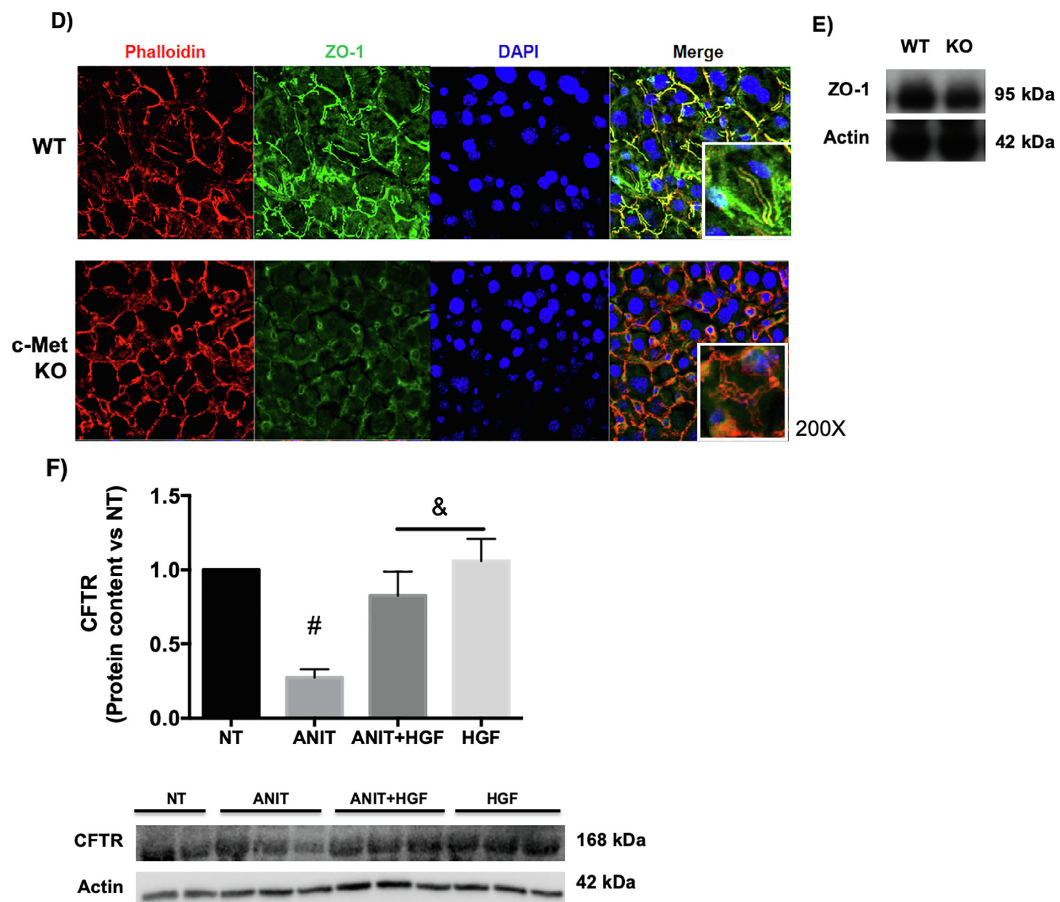


**Fig. 4.** (*continued*)

glutathione or glucuronic acid, such as glucuronide bilirubin (Fig. 6). *Abcc2*/Mrp2 and *Abcb11/Bsep* mRNA levels increased in ANIT alone group, probably as an adaptive mechanism in cholestasis, while the co-treatment with HGF counteracted these changes. On the other hand, the mRNA levels of the basolateral transporter *Abcc3*/Mrp3 and *Abcc4*/Mrp4 increased in co-treated animals (Fig. 6), and interestingly, *Abcc4*/Mrp4 increased as well in HGF alone treatment.

Improvements in the expression of *Abcb11*/Bsep at the canalicular level and that of *Abcc3*/Mrp3 and *Abcc4*/Mrp4 at the basolateral level may help to explain the improved depuration of bile salts and bilirubin in the co-treated group (Fig. 2A), and the lower damage at the histological level (see Fig. 2D).

## 4. Discussion

HGF exerts protective effects in different liver diseases, such as fibrosis [28], hepatocarcinogenesis [36], and drug-induced hepatotoxicity [8], among others [10]. An improvement in survival response has also been documented in other organs, such as the pancreas [14] and lungs [13], particularly by a mechanism dependent on ROS modulation.

The pathogenesis of cholestatic diseases remains poorly studied, despite the increase in incidence [6,37,38]. Consequently, new therapeutic options are required to counteract these disorders. In the present work, we aimed to evaluate the protective role of HGF in intrahepatic cholestasis by using ANIT as a model of cholestatic hepatobiliary damage.

ANIT-induced cholestasis leads to the accumulation of bile salts in the liver parenchyma, and this represents a second hit that contributes to perpetuation of the disease. Although the mechanism of ANIT-induced cholestasis has not been yet fully elucidated, a recent study showed that ANIT can impair expression of a number of hepatobiliary transporters involved in bile acid hepatic handling in sandwich-cultured rat primary hepatocytes [39], in line with our results here “in vivo”. This seems to involve bile salt-dependent AMPK activation via the ERK1/2-LKB pathway, and the further repression of farnesyl X receptor, a master nuclear receptor that regulates bile salt homeostasis [40]. A number of adaptive mechanisms are activated in an attempt to attenuate bile acid accumulation and protect hepatocytes against further injury, mainly induction of basolateral export pumps that extrude these compound to blood, thus allowing their further urinary excretion as an alternative depuration pathway [41]. Oxidative stress seems to play a key role in ANIT-induced cholestasis. In line with our results here, lipid peroxidation, a surrogate marker of oxidative stress, increased rapidly (from 12 h onwards) after “in vivo” ANIT administration, through mechanisms involving, at least in part, a pro-oxidant unbalance by ROS derived from neutrophils infiltrated into the liver tissue [42] and activation of NADPH oxidase 4 expression [43] on one hand, and impairment of antioxidant defenses, including impaired activity of superoxide dismutase and catalase activity [44] on the other hand. Oxidative damage can represents, therefore one of the main first hit that mediates ANIT cholestatic effect on hepatocytes, as oxidative stress rapidly triggers cholestatic mechanisms, including actin

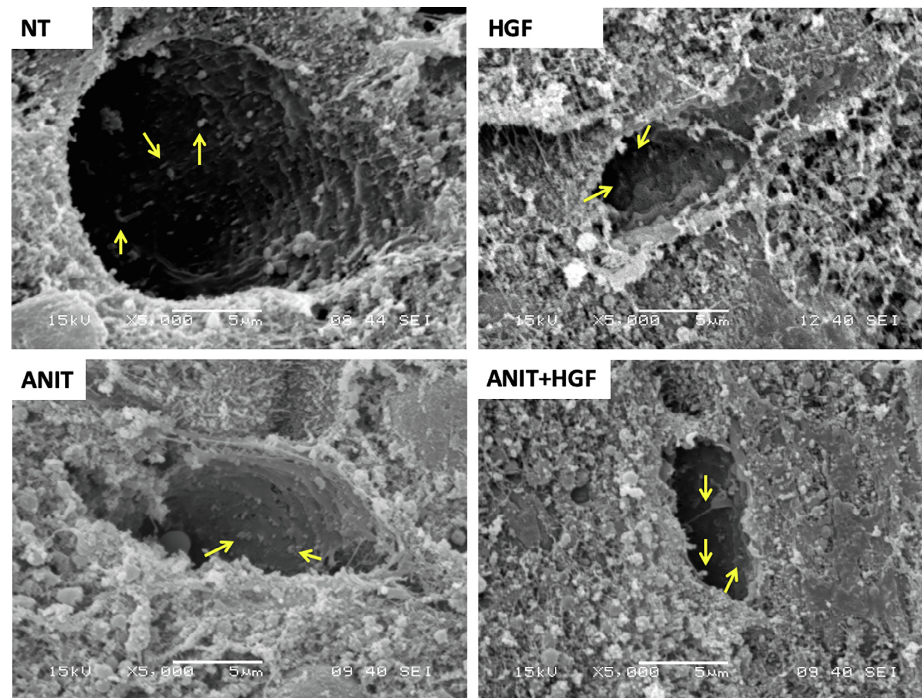


**Fig. 5.** HGF preserves the cholangiocellular primary cilium. Scanning electron micrograph from livers showing cilia from bile canaliculi (arrows). The images are representative of at least three animals.

disorganization followed by endocytic internalization of the canalicular export pumps Bsep [45], and Mrp2 [46], and thigh-junctional disruption [45]; this leads to a second hit associated with the rapid bile salt accumulation, which may further aggravate oxidative damage, since bile salts are strong pro-oxidant compounds that can promote ROS-mediated hepatocellular apoptosis and necrosis [47], as observed in our cholestatic model. It is therefore not surprising that antioxidants has anticholestatic effects on ANIT-induced cholestasis, as was shown for α-tocopherol [44], paeoniflorin [43], SRT1720 [48], and melatonin [44,49], among many others antioxidant compounds. Interestingly, some of these compounds, such as melatonin [50], SRT1720 [48], 18ß-glycyrrhetinic acid [51], paeoniflorin [52], and dioscin [53], exert antioxidant and anticholestatic effects in ANIT-induced cholestasis and, simultaneously, Nrf2 activation, thus suggesting a causal association between both phenomena. It is therefore highly likely that HGF had exerted anticholestatic effects by activation of Nrf2, since this nuclear receptor is a main downstream mediator of this growth factor [16]. Evidences that a "pure" Nrf2 activation can exert anticholestatic effects are however currently lacking. Oltipraz, a well-known Nrf2 activator, had however anticholestatic effects in Nrf2-null mice [26], thus suggesting that this compound protects livers from ANIT-induced hepatotoxicity via a Nrf2 pathway-independent pathway, perhaps involving constitutive androstane receptor (CAR), a nuclear receptor with anticholestatic properties that is also activated by oltipraz [54]. Therefore, our evidence here on the anticholestatic effects of HGF, a pleiotropic cytokine that selectively activates Nrf2 as we previously reported [16], adds supports to the anticholestatic effects of Nrf2 *per se*.

The possible mechanisms by which HGF would afford anticholestatic effects via Nrf2 in our model are multifactorial. We have shown that HGF as a master regulator of the cellular redox state, since it displays mechanisms mediated by Nrf2 directed to both the induction of antioxidant enzymes [9,10] and the abrogation of pro-oxidant systems, such as NADPH oxidase, which are involved in ANIT-induced cholestasis [43]. Our results clearly show a remarkable increment in ROS content induced by ANIT, and the significant normalization afforded by HGF (Fig. 3), thus suggesting that this cytokine abrogate indeed this pro-oxidant first hit triggering the cholestatic phenotype.

Although this effect likely involved the Nrf2-mediated induction of antioxidant proteins, Nrf2 also induces phase II and III detoxification processes, leading to the elimination of noxious compounds, which, in addition, exerts an indirect antioxidant effect by removing pro-oxidant entities [16,17].

As for phase II detoxification systems, this transcription factor positively modulates the expression of hepatocellular transporters impaired by ANIT, or that of basolateral export pumps that help to maintain low the intracellular levels of bile salts despite the secretory failure; this abrogates the bile salt-mediated second hit, which leads to hepatocellular death, among other deleterious effects. In the present work, we observed an increment in the basolateral bile salts transporter Mrp3 and Mrp4 hepatic expression due to HGF treatment in mice co-treated with ANIT (Fig. 6), thus supporting this content. As shown by studies in Mrp3 and Mrp4 knockout mouse models, bilirubin (mainly as glucuronide conjugates), are the preferred substrate for Mrp3, whereas bile acid conjugates are the preferred substrate for Mrp4 in the cholestatic liver[55]. Both endogenous bile salts [56], and bilirubin [57], particularly in their unconjugated (apolar) form, are highly cytotoxic substances, leading to cell death by necrosis and apoptosis through different mechanisms, involving mitochondrial dysfunction with further oxidative stress generation, disorganization of the plasma membrane, and reticulum endoplasmic stress, among other deleterious effects.

On the other hand, HGF exerts a differential effect on canalicular transporters by transcriptionally decreasing Bsep and Mrp2 expression (Fig. 6), thus suggesting redirection of these compounds to the bloodstream [58,59]. Lack of induction of these transporters and the resulting absence of choleretic effect of HGF may have important positive

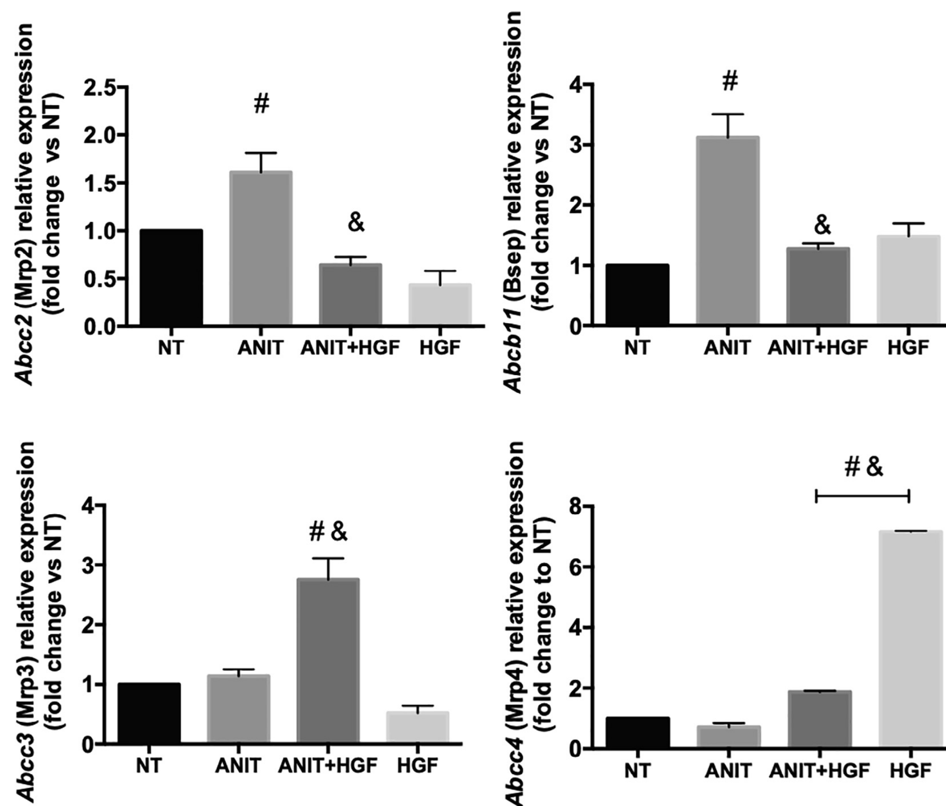


**Fig. 6.** HGF modulates efflux ABC transporter expression. Messenger RNA levels of the ABC transporters genes Abcc2, Abcb11, Abcc3 and Abcc4. Each bar represents median ± SEM. $^{\#}p < 0.01$ vs NT, $^{\&}p < 0.01$ vs ANIT.

therapeutic implications, particularly under biliary obstruction conditions, as occurs in our model [25], and in obstructive cholangiopathies in humans. This is consistent with findings in the Mrp2-deficient rat which presented protection in ANIT-induced cholestasis [60], it is clear that basolateral elimination of ANIT instead of the biliary route avoids the cholestatic damage as observed in both the HGF-treated mice and the Mrp2-deficient rat.

The choleretic, anticholestatic therapeutic bile acid UDCA, which induced selectively Bsep to increase bile acid-dependent bile flow in ANIT-induced cholestasis, aggravated liver injury when the bile ducts were obstructed [61]. This is most likely a consequence of the biliary infarcts of Hering canals due to the increased intraluminal hydrostatic pressure in obstructed ducts, which leads to extravasation and direct contact of toxic bile with hepatocytes [62]. Suggestively, the clinical benefit of UDCA in the treatment in obstructive cholangiopathies, such as PSC and late-stage primary biliary cholangitis, are limited, when not detrimental [63]. Therefore, therapeutic options that redirects biliary toxic compounds into urine without generating neither choleresis nor increase in bile salt output, as showed here for HGF (Fig. 2 E and F), may be particularly helpful in obstructive cholangiopathies.

HGF seems to have not only favorable functional impact on the cholestatic injury, but also important effects on structural features, such as tight junctional structures, ultimately impact on the secretory function. ANIT is known to alter tight junctional-dependent paracellular permeability as soon as 16 h after ANIT administration [25,64], leading to dissipation of biliary osmotic gradients via the paracellular shunt and, consequently, bile flow impairment. We have shown here that these functional changes in the tight-junctional barrier has a clear structural correlate. Gross alterations in localization of ZO-1, an F-actin–binding protein that localizes to tight junctions and connects them to actin cytoskeleton, were observed, as a loss of its characteristic ‘double rail’ array (Fig. 4). Since a reduction in actin expression was reported to occur at 48 h of ANIT exposure [65], ZO-1 disorganization can be a direct consequence of the loss of its anchor protein, actin. This does not rule out the possibility that, besides, disorganization of F-actin, a common feature under oxidative stress conditions [45], may have also contributed to ZO-1 disorganization. Even, actin oxidation without actin disorganization may be involved, since oxidative changes in this protein usually inhibits its association with actin-binding proteins, like ZO-1 [66]. Whatever is the underlying mechanism, HGF fully prevented ZO-1 disorganization, opening the possibility that the cytokine also prevents the tight-junction impairment at a functional level.

Our study also provides evidence of cholangiocytes damage, as judged by GGT elevations (Fig. 2A), a cholangiocellular but not hepatocellular enzyme that is induced and removed by biliary bile salts in obstructed ducts [67], the decreased activity of the cholangyocyte chloride channel CFTR (Fig. 4D), and the shortening/disappearance of the primary cilia (Fig. 5). Bile duct damage in ANIT-induced cholestasis occurs by the reversible formation of an unstable glutathione-ANIT conjugate within hepatocytes, which partially dissociates once secreted into bile via Mrp2 due to the alkaline milieu, yielding glutathione and high, toxic levels of unconjugated ANIT in bile [60]. The toxic effects seems to be mediated by highly reactive products formed from the thiocyanate moiety of the molecule after dissociation [68], that can interact with sulfhydryl groups of cholangiocellular surface proteins; damaged proteins are then endocyted and transported to the endoplasmic reticulum, where they initiate of the unfolded protein response and further oxidative stress [68].

The protective effect of HGF at this level may involve accelerated exportation of the glutathione-ANIT conjugate into blood and its further

urinary excretion via induction of Mrp4, which is an efficient glutathione S-conjugate transporter[69], or direct antioxidant effects of HGF on cholangiocytes, thus protecting them from ANIT-induced oxidative stress and cholangiocyte death. In line with this view, the HGF receptor c-Met seems to be expressed in normal cholangiocytes, as inferred for its readily apparent expression in the cell line of normal, immortalized cholangiocytes H69 [70]. Our findings that CFTR is donwregulated in ANIT-intoxicated animals and that its expression is restored by HGF are both novel and may have profound physiopathological and therapeutic implications. As part of its harmful effects on the biliary tree, ANIT induces a strong inflammatory response at this level, and CFTR was shown to be downregulated by proinflammatory cytokines in epithelial cells [71], probably thorough a mechanisms involving oxidative stress [72]. The decrease in CFTR activity may contribute to ANIT-induced cholestasis by impairing the bicarbonate-rich choleresis due to lack of luminal chloride to be exchanged by bicarbonate via the AE2 exchanger, and this may produce *i*) decreased ductular bile flow leading to inspissation and mucus plugging, thus aggravating the obstructive process, and *ii*) lack of the so called “bicarbonate umbrella”, which maintain bile salts in their ionic, non-diffusible form, thus impeding cholangiocyte from being attacked by these toxic compounds [73]. In addition, loss of cholangiocyte primary cilium induced by ANIT, as we have shown here (Fig. 5), can contribute to the pathogenesis of the disease. Cholangiocytes proliferate under an obstructive cholestatic condition, as an adaptive response to the liver injury caused by bile salt overload. This adaptive mechanism consistent of expansion of bile ducts and induction of liver progenitors that can differentiate into both hepatocytes and cholangiocytes to replace the damaged cells [74]. The primary cilia are the chemosensors that mediate this adaptive mechanism triggered bile salts, since they express the bile salt receptor TGR5 [74,75]. Therefore, by counteracting cilium damage, HGF would allow bile ducts to trigger this adaptive, reparative process.

In addition, inflammatory infiltrate presented by damaged tissues [76] was not found in ANIT + HGF liver sections (Fig. 2D), suggesting that HGF could also act as a possible immunomodulatory agent, as previously suggested [13,77].

Although all these protective effects were induced by supraphysiological levels of HFG, even physiological levels of HGF seems to afford certain anticholestatic effects. As reported previously by our group [11], mice fed with high cholesterol diet displayed high serum bile salt levels, but the effect was more pronounced in Met KO animals. Indeed, cholesterol-fed Met KO mice had an increased bilirubin and ALP serum levels. Also, they presented a decrease in FXR levels, a key transcription factor for bile salt homeostasis whose impaired expression has been suggested to be a pathogenic factor in many cholestatic diseases, and that foster liver regeneration and growth properties that help to repair liver damage [78].

We also reported that one of the main hepatopathies developed by the lack of c-Met in the c-Met KO mouse was cholestasis. The global transcriptomic analysis of these mice exhibited defective signaling pathways associated to normal hepatobiliary function, such as bile acid biosynthesis, glutathione metabolism, and Nrf2-mediated oxidative stress response [11]. These results further support our contantion that HGF, given at supraphysiological levels for pharmacological purposes, can counteract pathogenic mechanims in cholestatic diseases when their pathomechanisms involves deficit in any of these HGF-regulated pathways, by counteracting this deficiency with an strengthened HGF signaling.

An interesting finding we made was that the absence of c-Met in MetKO animals produced a disruption in the canalicular structure (Fig. 4D). The observed effect is only structural, since no change in the content of ZO-1 was observed (Fig. 4E). These results confirm that HGF/c-Met are of main importance for proper canalicular structure. β-Catenin, one of the canonical efectors of HGF/c-Met signaling, is a key regulator of canalicular morphology and function, and liver-specific loss of this protein is directly associated with imperfect bile canalicular morphology, a physiological dysfunction leading to cholestasis [30].

In conclusion, our study highlights a novel aspect of HGF as anti-cholestatic agent, which protects the liver against ANIT-induced cholestatic damage. This points HGF and its relevant downstream mediators as a possible therapeutic tool for cholestasis treatment.

## CRediT authorship contribution statement

**Soraya Salas-Silva:** Investigation, Methodology. **Arturo Simoni-Nieves:** Investigation. **María Valeria Razori:** Investigation. **Jocelyn López-Ramirez:** Investigation. **Jonatan Barrera-Chimal:** Data curation. **Roberto Lazzarini:** Investigation. **Oscar Bello:** Investigation, Methodology. **Verónica Souza:** Investigation, Validation. **Roxana U. Miranda-Labra:** Investigation, Validation. **María Concepción Gutiérrez-Ruiz:** Data curation, Validation, Writing - review & editing. **Luis Enrique Gomez-Quiroz:** Methodology, Data curation, Funding acquisition, Writing - original draft, Writing - review & editing. **Marcelo G. Roma:** Data curation, Funding acquisition, Writing - original draft, Writing - review & editing. **Leticia Bucio-Ortiz:** Funding acquisition, Methodology, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## 11.3 Artículo de revisión asociado al trabajo de investigación doctoral

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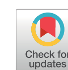
Review article

# Cholangiocyte death in ductopenic cholestatic cholangiopathies: Mechanistic basis and emerging therapeutic strategies



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ABSTRACT

Among hepatic diseases, cholestatic ductopenic cholangiopathies are poorly studied, and they are rarely given the importance they deserve, especially considering their high incidence in clinical practice. Although cholestatic ductopenic cholangiopathies have different etiologies and pathogenesis, all have the same target (the cholangiocyte) and similar mechanistic basis of cell death. Cholestatic cholangiopathies are characterized, predominantly, by obstructive or functional damage in the biliary epithelium, resulting in an imbalance between proliferation and cholangiocellular death; this leads to the progressive disappearance of bile ducts, as has been shown to occur in primary sclerosing cholangitis, primary biliary cholangitis, low-phospholipid-associated cholelithiasis syndrome, cystic fibrosis-related liver disease, and drug-induced ductopenia, among other biliary disorders. This review summarizes the features of the more common ductopenic syndromes and the cellular mechanisms involved in cholengiocellular death, with focus on the main forms of cholangiocyte death described so far, namely apoptosis, autophagy, necrosis, and necroptosis. It also emphasizes the importance to study in depth the molecular mechanisms of cholengiocyte death to make possible to counteract them with therapeutic purposes. These therapeutic strategies are limited in number and efficacy at present, and this is why it is important to find complementary, safe strategies to stimulate cholangiocellular proliferation in order favor bile duct replenishment as well. Successful in finding appropriate treatments would prevent the patient from having liver transplantation as the only therapeutic alternative.

## 1. Introduction

Cholestatic diseases are frequently dismissed as compared to others liver diseases, such as viral hepatitis, nonalcoholic steatohepatitis, and hepatocellular carcinoma, among others. However, most of chronic hepatopathies arise from, or progress towards cholestatic cholangiopathies. In the first three months of 2018, 4798 liver transplants were carried out in USA, with cholestatic cholangiopathies being one of the main causes of this treatment (United Network for Organ Sharin Website; https://unos.org).

Cholagiopathies result from a set of disorders associated with the biliary tract. They represent an actual therapeutic challenge, due to the complex function and anatomy disposition of bile ducts. Actually, there are no appropriate experimental models to study these disorders excluding, perhaps, the bile-duct ligation model [1,2] and the Mdr2-deficiente mouse model [3], which mimic obstructive cholelithiasis and low phospholipid-associated cholestasis secondary leading to sclerosing cholangitis, respectively.

The biliary tree is a highly dynamic structure with cells specialized in bile secretion, bile acid reabsorption, drug metabolism, and immune regulation [4–6]. It is composed of bile ducts, which extend from the small canals of Hering to the large extrahepatic bile duct (Fig. 1) [4].

The biliary epithelial cells form a physical barrier, and act as the first line of defense against the potentially cytotoxic components of bile [7]. Therefore, bile formation and modification require not only functional but also structural integrity of hepatocytes and cholangiocytes [5].

Biliary tree homeostasis is achieved through a balance between cell

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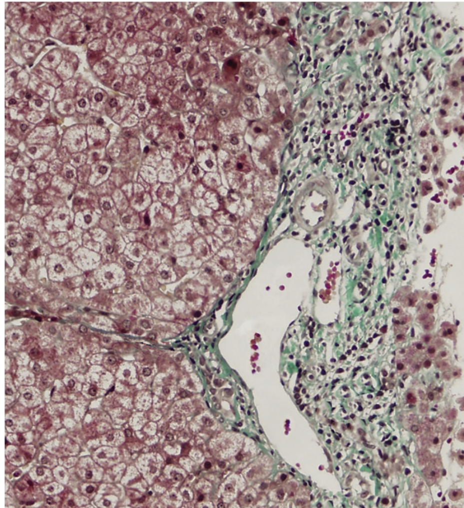


**Fig. 1.** Liver biopsy specimens of a patient with ductopenia. Portal trial showing absence of interlobular biliary ducts and lymphoplasmocytic infiltrate in a patient with PBC. Piecemeal necrosisis is also observed (right, low corner). Under normal conditions, biliary ducts should be observed in a space lower than two artery diameters. Masson, x40.

death and regeneration [8,9]. When this balance is lost due to exacerbation of cell death or impaired proliferation, the biliary system is affected in several ways that may lead to ductopenia and development of certain hepatopathies, collectively called "ductopenic cholangiopathies". These chronic and progressive liver disorders, characterized by the clinical syndrome of cholestasis due to an affected biliary tree, are responsible for the significant morbidity and mortality rates among pediatric and adult populations.

In this review, we describe the most frequent ductopenic cholestatic cholangiopathies by analyzing their general clinical characteristics and physiopathogenesis mechanisms, and explain in greater detail the mechanisms of cholangiocellular death that lead to these liver diseases. Finally, some hints for therapeutics to limit the ductopenic injury are provided, based either on cholangiocyte protection against these mechanisms of cell death or in the stimulation of cholangiocyte proliferation.

## 2. Cholestatic cholangiopathies

Cholestasis (literally, "a standing still of bile") is a syndrome originated from a disruption in bile production or flow through the biliary tree, resulting in a decreased amount of bile reaching the duodenum. The cause for this alteration can be a primary impairment in the capacity of hepatocytes or cholangiocytes to generate bile (functional or metabolic cholestasis), or a mechanical impairment hindering or impeding the bile transit (obstructive cholestasis) [10].

Cholestatic cholangiopathies are associated with a functional, obstructive, or mixed damage to the biliary tree, resulting in cholestasis. This disorder is the major determinant of most chronic cholestatic disorders.

All cholestatic cholangiopathies share, either as a primary or a secondary cause, an imbalance between cholangiocyte proliferation and death rate, associated with portal inflammation and fibrosis. Such alterations may be the main primary cause of the cholestatic disorder or a secondary manifestation of a primary hepatocellular dysfunction, which exacerbates the initial cholestatic phenomenon by extending the hepatocellular dysfunction to the cholangiocyte [11].

Cholestasis may arise from a primary obstruction in the bile ducts, but it also develops after the onset of ductopenia [8,9], which may induce loss of functional cholangiocellular mass and a biliary obstruction secondary to cellular detritus build-up and to the fibrosis caused by the inflammatory response thereto [12]. This damage induces the release of cytokines and pro-inflammatory mediators by cholangiocytes or other cell types, which stimulates cell death and proliferation responses, fibrogenesis, and impairment of the biliary epithelium transport function [11].

Chronic cholestatic cholangiopathies are the main cause of liver disease, cirrhosis, and progressive failure, eventually requiring liver transplant for the patient to survive. Some cases of cholestatic diseases are related to mutations in genes encoding proteins that are expressed in the bile canaliculus, and that are important for normal bile flow generation [13].

Cholangiopathies develop when the bile epithelium is damaged, either by infectious agents, autoimmune or genetic disorders, toxic compounds, or ischemia, which gives rise to several abnormalities in the bile duct, as observed in prototypical cholangiopathies such as primary biliary cirrhosis, cystic fibrosis, or biliary atresia [11].

The damage in the bile duct can be attributed to different causes, namely (1) the direct toxic effect of unmetabolized drugs on cholangiocytes (or reactivity of one of their metabolites) [14], (2) the immediate immune response triggered by cholangiocellular damage (e.g., due to an infectious agent or toxin), or autoimmunological causes associated with loss of tolerance to cholangiocellular antigens [6], and (3) a disruption in the protection mechanisms of the cholangiocyte against potentially toxic biliary compounds (e.g., due to a decrease in biliary phospholipids or an alteration in ductular bicarbonate excretion, two protective factors against cytotoxicity of tensioactive bile salts) [7]. Biliary complications, usually of ischemic nature [15], also constitute one of the main causes of morbidity and mortality after liver transplantation [16].

## 3. Ductopenic cholestatic cholangiopathies

Accelerated chollangiocellular death causes ductopenia, which is defined as the absence of interlobular bile ducts in no less than 50% of the portal triads. Its progression leads to vanishing bile duct syndrome (VBDS) when the bile ducts have virtually disappeared (Fig. 2) [8].

Among the different ductopenic cholestatic cholangiopathies, primary sclerosing cholangitis, primary biliary cirrhosis, biliary atresia, phospholipid decrease-associated cholestasis, cystic fibrosis, and drug-induced cholangiopathy are the more relevant ones [17], and they will be briefly described below.

### *3.1. Primary sclerosing cholangitis (PSC)*

Primary sclerosing cholangitis is a chronic cholestatic autoimmune disease of unknown etiology, in which bile ducts are progressively disorganized due to the permanent presence of fibrosis and dilation of the intrahepatic and extrahepatic bile ducts due to multifocal stenosis, eventually leading to the development of biliary cirrhosis and/or liver failure [18]. It has a prevalence of 0–16.2 cases per 100,000 inhabitants [19].

The characteristic histological changes of PSC are concentric periductal fibrosis (also known as onion skin), and fibro-obliteration of medium-sized or larger bile ducts [6,20]. PSC can develop in 4 stages. At stage 1, or portal stage, lesions are minor; portal edema, lymphocyte infiltration into bile ducts, and non-destructive cholangitis appear in this stage. At stage 2, periportal fibrosis develops. At stage 3, there is septal fibrosis with major damage to the bile ducts, inducing their progressive disappearance (ductopenia). Lastly, cirrhosis is developed at stage 4 [21,22].

PSC etiology is currently unknown, but several pathophysiological mechanisms have been suggested. Defects in the mechanisms that protect against the toxicity of tensioactive bile salts have been proposed

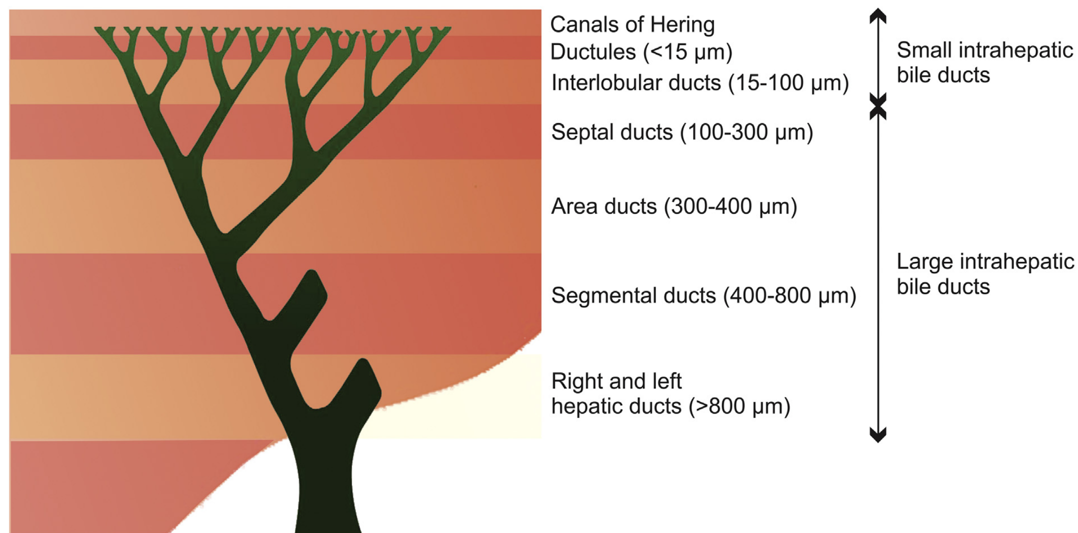



**Fig. 2.** Schematic representation of the intrahepatic biliary track. The intrahepatic biliary tree is a network of ducts that initiates in the canals of Hering, and gradually merges into an arrangement of interlobular, septal, and major ducts that finally coalesces to form the extrahepatic bile ducts.

as key actors in its development [7]. Release of intestinal pro-inflammatory microbial components into portal circulation (e.g., lipopolysaccharides), capable of triggering an innate immune response, has also been proposed, as well as the possibility of an antigenic factor of intestinal microbial origin [18]. Hepatic recruitment of T lymphocytes derived from intestine, due to overlapping in the pattern of adhesion molecules between intestine and bile ducts, has also been suggested [23]. T cells would then reach the portal area and the peribiliary space, thus inducing focal, fibro-obliterative lesions. Progressive periductal fibrosis, chronic inflammation, and ischemic atrophy of the biliary epithelium cause ductopenia, cholestasis, and obstructive stenosis, thus leading to secondary biliary cirrhosis [24].

Antibodies, such as perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), anti-nuclear antibodies (ANA), and smooth muscle antibodies (SMA) have been reported to be present in this disease [21]. This may reflect, in part, the response of B lymphocytes to antigens of intestinal origin (for pANCA) [25] or genetic causes associated with mutations in the *major histocompatibility complex* (MHC) responsible for antigen presentation, which are predominantly connected to autoimmune diseases related to the adaptive immunologic function; this may also explain the abnormal presence of several other autoantibodies (e.g., ANA and SMA) [26,27].

### 3.2. *Primary biliary cholangitis (PBC)*

Formerly known as ‘primary biliary cirrhosis’, PBC is an autoimmune disease [28] that selectively destroys small, interlobular, and septal bile ducts [6,20,29]; this leads to cholestasis, with increased levels of serum alkaline phosphatase, γ-glutamyltransferase, and bilirubin [30]. This hepatopathy is associated with progressive ductopenia and fibrosis, potentially leading to cirrhosis and, eventually, liver failure [28].

PBC predominantly affects middle aged or elderly women (1:9) [6,28], and it is the leading cause of ductopenia in adults [17]. PBC prevalence rates range from 1.91–40.2 per 100,000 inhabitants, and these values are increasing with time [19].

Histopathologically, PBC destroys intrahepatic bile ducts [6], with focal obliterations of the ducts and granuloma formation [21], inflammation due to infiltration of lymphocytes (CD4+ and CD8+) [17], macrophages, and other inflammatory cells [6]. Vacuolar degeneration and apoptosis of epithelial cells and disruption of the basement membrane is also observed [20]. This leads to the subsequent development of portal fibrosis and, eventually, cirrhosis [31]. More than 40% of PBC patients display ductopenia at their baseline biopsy [32].

Besides PBC, there are other forms of biliary cholangitis, which are secondary in nature. For example, it can represent a late manifestation of extended events of intrahepatic and/or extrahepatic bile duct obstruction (obstructive biliary cirrhosis) [33], or it may be due to other secondary causes of alteration of the bile ducts, e.g., those of infectious or ischemic nature [31], which are usually associated with cholangitis and acute pericholangitis [34].

Regarding pathogenesis, PBC is a prototypical autoimmune liver pathology. It is characterized by the presence of antimitochondrial autoantibodies (AMA) against the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC-E2) [6,35]. These autoantibodies evoke an inflammatory response characterized by infiltration of CD4+ ("helper") and CD8+ ("cytotoxic") T cells specific of mitochondrial antigens, in small and medium-sized bile ducts (i.e., larger interlobular and septal ducts). These autoantibodies are produced as a consequence of the systemic exposure of the PDC-E2 mitochondrial intracellular antigen caused by its release into the extracellular medium after cholangiocyte apoptosis [36]. In most tissues, PDC-E2 conjugates its sulfhydryl groups with glutathione (GSH) during apoptosis, rendering it antigenic in nature. However, the high levels of Bcl2 proapoptotic proteins that constitutively the cholangiocytes express inhibit this conjugation, thus exacerbating its antigenic effects [37,38]. PBC also has high serum IgM levels [28], possibly as a consequence of a decreased commutation of this kind of immunoglobulin into an IgG with similar antigenic specificity [38].

### 3.3. *Biliary atresia (BA)*

BA is a severe idiopathic neonatal cholangiopathy characterized by the rapid progression of fibrosis, obliteration, and destruction of the extrahepatic bile ducts during the first weeks of life [6,39–41]. The intrahepatic bile ducts loss their ramifications, a feature of mature ducts, resulting in cholestasis and neonatal hepatitis [42]. Histologically, the extrahepatic bile ducts exhibit extended fibrosis with inflammatory foci, whereas the intrahepatic ducts are hyperplastic, with variable inflammation and fibrosis, surrounded by giant multinucleated

hepatocytes. Approximately 30% of cases also suffer ductopenia, with narrowed interlobular ducts [43].

This pathology has a female predominance, especially in patients who also have spleen malformation [39]. During the course of the pathology, typical signs and symptoms of cholestasis appear, such as jaundice, pale stools, dark urine, portal hypertension, ascites, splenomegaly and, in certain cases, bleeding [42]. Biliary atresia is the most frequent hepatic cause of child death [44]. The main treatment for atresia is liver transplantation [39], which has improved patient survival [40,41].

Several factors are involved in the development of this disease, such as embryogenesis defects, abnormal fetal or prenatal circulation, viral infections, abnormal inflammatory response, genetic factors, autoimmunity, and environmental toxins [39]. It has been described that, akin to Alagille syndrome (a congenital ductopenic cholestatic cholangiopathy with similar characteristics to BA), a defect in the Notch signaling pathway, which controls transdifferentiation of hepatoblasts and mature hepatocytes into cholangiocytes, alters the expression of hepatic nuclear factor-1β (HNF-1β) [45,46]; this affects the repair mechanisms in postnatal life, due to the absence of reactive ductular cells and accumulation of hepatobiliary cells lacking HNF1β that cannot differentiate into a biliary phenotype [47].

### 3.4. Low-phospholipid-associated cholelithiasis syndrome (LPAC)

This pathological entity encompasses a group of liver diseases characterized by a variable defect in the biliary excretion of phospholipids. It is causally associated with mutations in the *multidrug resistance protein 3* (MDR3), a floppase encoded by the *ABCB4* gene that enables excretion into bile of phospholipids, such as phosphatidylcholine, by flopping them from the inner to the outer leaflet of the hepatocyte canalicular membrane, from where they are removed by action of luminal detergent bile salts [48–50]. The biliary excretion of phospholipids is crucial to protect both the canalicular membranes of hepatocytes and the apical membranes of cholangiocytes, as the phospholipids present in bile allow for the formation of mixed micelles composed of phospholipids, cholesterol and bile salts, thus protecting the epithelium against the detergent effect of free, monomeric bile salts in the biliary lumen [7,50]. Actually, hydrophobic bile salts have been demonstrated to cause necrosis and apoptosis in immortalized mouse cholangiocytes, and the effect is avoided by luminal phospholipids [51].

Since MDR3 mutations are varied in nature and have different functional impacts, they produce a wide spectrum of severity, depending on the degree of functional alteration [50,52]. In the most benign forms associated with mutations of limited functional impact, bile lithogenicity and cholesterol crystallization increase due to defects in cholesterol micellization, thus inducing obstruction of small bile ducts because of the formation of "biliary sludge"; even in its asymptomatic forms, this entity could be a predisposing factor for cholestatic conditions when potentially harmful factors are added, such as cholestatic hormones (e.g., endogenous estrogens and progesterone in cholestasis of pregnancy) or MDR3-inhibiting drugs (e.g., cyclosporine A, sirolimus, verapamil, and vinblastine) [50,52]. In its more aggressive forms associated with significant functional defects of MDR3, an overt, severe cholestatic disease develops, referred to as "progressive familial intrahepatic cholestasis type 3" (PFIC-3). This hereditary cholestasis occurs at a very young age, with a spectrum ranging from neonatal cholestasis to biliary cirrhosis in young adults. Its progression rate is associated with continuous exposure to detergent bile salt monomers. In homozygotes, a nonsense mutation in the MDR3 gene has been associated with ductopenia [53]. This pathology develops during childhood and adolescence, when the patients show typical cholestasis signs and symptoms, including jaundice, pale stools, hepatomegaly, and pruritus. Gastrointestinal bleedings and cirrhosis with portal hypertension may also occur. It has even been reported that MDR3 deficiency is a high-risk factor for developing hepatocarcinoma (HCC), and cholangiocarcinoma (CCA) has also been described in these patients [7,13,54].

Histologically, portal fibrosis and variable periportal fibrosis may be present, with progression to micronodular biliary cirrhosis, as well as ductular proliferation and infiltration of inflammatory cells, mostly lymphocytes; hepatocellular, canalicular, and ductular bilirubinostasis are also observed [13,54–56].

### 3.5. Cystic fibrosis (CF)-related liver disease

CF is a recessive autosomal disorder in the gene encoding the *cystic fibrosis transmembrane conductance regulator* (CFTR), an ATP-dependent cholangiocyte $Cl^-$ channel. CFTR insertion into apical membrane of cholangiocytes is mediated by secretin-induced cAMP elevations; the high luminal-to intracelular $Cl^-$ electrochemical gradient thus formed drives $HCO_3^-$ ductular excretion via the *anion exchanger 2* (AE2), which exchange $HCO_3^-$ by $Cl^-$ in an equimolar manner. Since AE2-mediated $HCO_3^-$ excretion is a chief determinant of ductular bile flow, dysfunction of CFTR may induce ultimately a decrease in ductular bile flow. Impairment of ductular bile flow provokes mucus plugging and further luminal obstruction in those bile ducts that cannot be drained normally [57,58]. Bile viscosity has been also verified to increase due to the high mucin content, and this may contribute to cholestasis [59], in part by promoting cholelithiasis [60]. The prevalence of CF-related liver disease ranges from 26% to 45% of CF patients. Portal hypertension without hepatocellular insufficiency is the main presentation of CF-related lived disease, and ductopenia in small portal tracts is observed frequently, in part associated with a diffuse obliterative portal venopathy [61].

In this pathology, production of the "$HCO_3^-$ umbrella," formed by the ductular secretion of $HCO_3^-$ promoted by secretin, is also affected. $HCO_3^-$ provides an alkaline environment along the apical surface of the bile epithelium that maintains bile salts in their ionic forms, thus preventing them from passively entering the cholangiocyte in their acidic, uncharged forms by non-ionic diffusion [7,62]. Since $HCO_3^-$ excretion is also a driving force of the ductular bile flow, failure to excrete this anion in CF also increases the intraluminal concentration of bile salts, thus elevating their cytotoxicity further [7,62]. A deficit of luminal protecting factors leading to cholangiocyte overexposure to bile salts may result in cholangiocellular damage, due to the pronecrotic and proapoptotic effects of these biliary compounds [63]. Actually, it has been proven that extracellular pH is critical for the hydrophobic bile salts to cause the cellular death of immortalized human cholangiocytes; in these cells, bile salts are slightly toxic when exposed to the bile pH (7.4), but the toxicity increases dramatically with small decreases in pH values (from 7.1 to 6.4), which is in the order of that expected to occur after a failure in ductular $HCO_3^-$ excretion [62,64]. In response to the ductular damage induced by these pathological mechanisms, a release of inflammatory cytokines occurs that leads to chronic portal inflammation; depending on the individual's immunogenetic basis and other concurrent factors, this may progress to focal biliary cirrhosis [64].

Neonatal cholestasis is an early manifestation of CF-related liver disease, which results in obstruction of the extrahepatic bile ducts; this could be one of the first manifestations of this pathology [57,58]. Up to 10% of CF patients develop cirrhosis in their first decade of life, progressing from focal biliary cirrhosis of the non-uniform portal tract to multilobular cirrhosis and portal hypertension [65], which could lead to liver failure and encephalopathy [58].

### 3.6. Drug-induced cholangiopathy

Many toxic drugs or their reactive metabolites can cause ductopenia and VBDS (Table 1).

These compounds may cause ductopenia mainly through the following pathomechanisms:

**Table 1**
Drugs that may induce ductopenic cholangiopathies.

| Aceprometazine | Co-trimoxazole | Norandrostenolone |
|---|---|---|
| Ajmaline | Cromolyn | Phenylbutazone |
| Amineptine | Cyamemazine Cyclohexyl propionate | Phenytoin |
| Amitriptyline | Cyproheptadine | Prochlorperazine |
| Amoxicillin/ clavulanic acid | D-penicillamine | Terbinafine |
| Ampicillin | Diazepam | Tetracyclines |
| Azathioprine | Erythromycin | Tiabendazole |
| Barbiturates | Estradiol | Tiopronin |
| Carbamazepine | Flucloxacillin | Trifluoperazine |
| Carbutamide | Glibenclamide | Tolbutamide |
| Chlorothiazide | Glycyrrhizin | Trimethoprim/ sulfamethoxazole |
| Chlorpromazine | Haloperidol | Troleandomycin |
| Cimetidine | Ibuprofen | Xenalamine |
| Ciprofloxicin | Imipramine | |
| Clindamycin | Methyltestosterone | |

1. Direct attack to cholangiocytes by drugs, or their toxic metabolites, once they have been excreted into bile.
2. Attack to cholangiocytes induced by drugs mediated by immunity, through the formation of immunogenic complexes between the drug (acting as a hapten) and endogenous proteins.

#### 3.6.1. Direct drug attack to cholangiocytes

Toxic drugs or their reactive metabolites can cause ductopenia when they reach the cholangiocyte after being excreted into bile. Frequently, they are electrophilic or nucleophilic chemical products (or even free radicals) capable of promoting a series of chemical reactions that damage the biliary epithelium through multiple mechanisms, such as loss of antioxidant defenses, GSH depletion, and covalent binding to proteins, lipids, and nucleic acids [66].

A prototypical compound that acts via this mechanism is α-naphthylisothiocyanate (ANIT). This cholestatic agent selectively damages cholangiocytes from small and large ducts, thus causing cholangitis and intrahepatic biliary obstruction due to development of biliary sclerosis and progressive destruction of interlobular ducts. Coexistent apoptosis and proliferation are present in small and large ducts [67]. Apoptosis may stimulate cholangiocyte proliferation to compensate for the loss of mass and ductal function, but unlike hepatocytes, the limited proliferative capacity of cholangiocytes is insufficient to prevent ductopenia [68]. The bile duct damage induced by ANIT requires its hepatocellular conjugation with GSH and the subsequent biliary excretion of this metabolite; ANIT forms an adduct of labile GSH within hepatocytes, which dissociates in bile due to the medium alkalinity after being transported by Mrp2 [69]. The bile duct injury is produced mainly through apoptotic mechanisms mediated by ANIT-induced oxidative stress [70].

According to reports with animal models, the hepatotoxic compound carbon tetrachloride ($CCl_4$) also damages the biliary epithelium through oxidative stress-mediated apoptotic mechanisms, but it affects selectively large cholangiocytes, which produces proliferation of small duct cholangiocytes [71]. In this case, $CCl_4$ is converted in hepatocytes into the trichloromethyl free radical ($\cdot CC_3$) by the cytochrome P450 isoform CYP2E1, followed by excretion of free radical adducts into bile [72].

Similar mechanisms may apply for several other toxic compounds. For example, flucloxacillin, a ß-lactam antibiotic of the isoxazolyl-penicillin family, causes cholestasis associated with VBDS [73], in which toxic metabolites are generated after CYP3A4-mediated metabolism (e.g., 5′-hydroxymethyl flucloxacillin), which damage cholangiocytes after biliary excretion [74].

#### 3.6.2. Immunologic attack to cholangiocytes by haptenization

As the liver metabolizes the drug, reactive metabolites can be produced, which may react with cellular macromolecules to form stable conjugates that trigger an aberrant immune response against self-proteins, thus forming autoantigens (haptenization) [75,76]. More commonly, haptens become immunogenic by forming adducts with proteins, either on the N-terminal domain (hydroxyalkylation) or on the C-terminal domain (oxoalkylation) [77]. Like in the molecular mimicry phenomenon, this immune response may also lead to reactivity against unmodified self-antigens, leading to immune tolerance breakdown and chronic autoimmunity [78].

## 4. Immunological mechanisms of ductopenic damage in cholangipathies

The mechanisms of immune-mediated ductular injury in cholangiopaties are common to most cholangiopathies. They are mainly related to the adaptive immune response mediated by CD4+ ("helper") T cells and CD8+ ("cytotoxic") T cells, damaging the cholangiocyte through highly specific T-cell receptors; the latter induce cholangiocellular death through apoptosis, by releasing perforin and granzyme B [79] (Fig. 3). For this immune response to occur, the antigenic moiety of the modified self-antigen needs to be displayed by an antigen-presenting cell (usually a dendritic cells) together with a *class-II MHC* (MHC-II) molecule, leading to a CD4+, T cell-induced immune response. Kupffer, hepatic stellate cells, and liver sinusoidal endothelial cells are all typical antigen-presenting cells in liver [80]. However, cholangiocytes themselves can also act as antigen-presenting cells in the inflammatory context [81], by aberrantly expressing MHC-I, MHC-II, and *intercellular adhesion molecule 1* (ICAM-1). This is due to the transcriptional effects of pro-inflammatory cytokines, such as interpheron-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) [82], thus increasing the possibility of activating "naïve" T cells, and initiating the cellular immune response [83].

Whatever the antigen-presenting cells are, activation of naïve CD4+, helper T cell after antigen presentation leads to CD4+ release of IL-2, followed by IL-2-induced autocrine clonal expansion. Active CD4+ lymphocytes stimulate, via IL-2 as well, the cell-mediated immunological attack of cholangiocytes by generating, activating, and allowing persistence of CD8+, cytotoxic T lymphocytes and natural killer (NK) cells, two cytolytic effector cells. These T lymphocytes bind to the cholangiocyte plasma membrane via the MHC-II/antigen complex or NKG2D-ligands, respectively [84], and release granzyme B and perforin, which induce apoptosis and necrosis in cholangiocytes [79]. In addition, soluble and plasma-membrane forms of *tumor necrosis factor-related apoptosis-inducing ligand* (TRAIL) and FasL from NK and maprophages interact with their respective receptors, TRAILR and Fas, expressed in cholangiocytes, and reinforce the apoptotic process [85]. Cholangiocytes also express constitutively low levels of leukocyte adhesion molecules, such as *class-I major histocompatibility complex* (MHC-I) and ICAM-1, but these molecules are upregulated during the inflammatory response [82]. Cholangiocytes can also secrete chemokines such as CXCL16 [86] or overexpress *monocyte chemotactic protein-1* (MCP-1) [87], which also promote inflammation by recruiting monocytes and T lymphocytes into portal tracts. MCP-1 upregulation seems to involve the epigenetic effects of micro-RNA miR-873-5p, whose circulating levels are increased in cirrhotic and cholestatic patients [88]; miR-873-5p targets glycine *N*-methyltransferase, the most abundant methyltransferase and master regulator of transmethylation flux involved in global epigenetic changes in liver [88].

Subsequently, an innate immunological response is produced, induced by the extracellular release of *danger-associated molecular patterns* (DAMPs) by the damaged cholangiocytes. DAMPs activate granulocytes and "natural killer" (NK) cells and target directly the cholangiocyte, via TLR receptors present in all these cells, able of recognizing and being activated by DAMPs [82,89]. DAMPs mimic immunological effects of

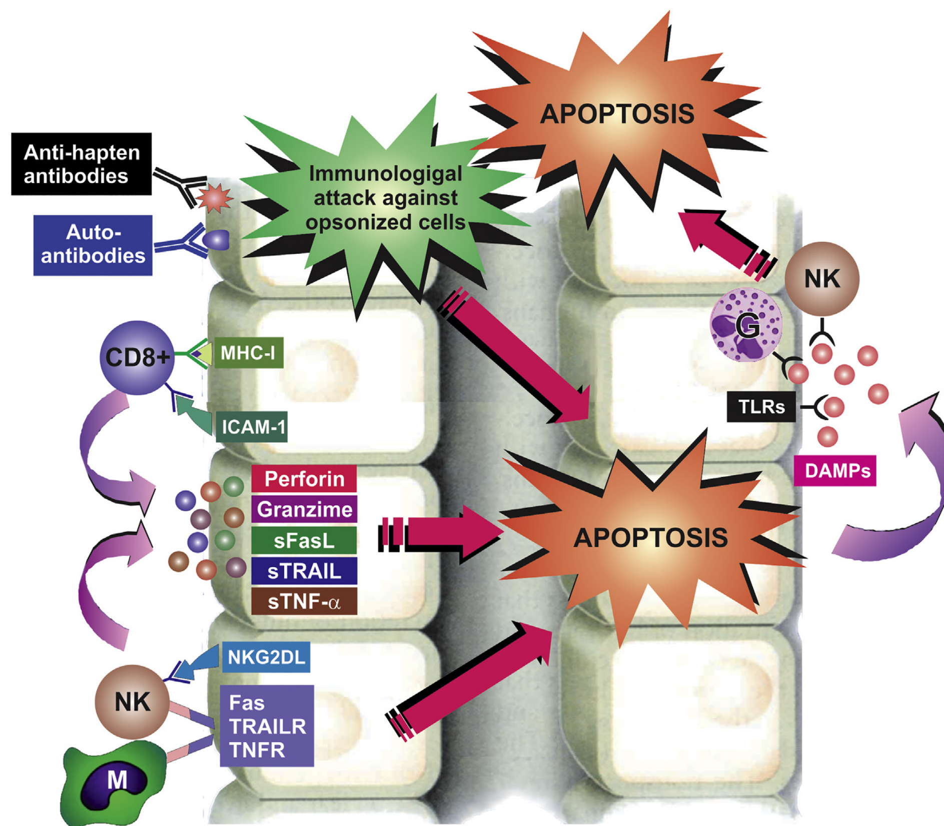



**Fig. 3.** Immunological mechanisms of ductopenic damage. Cytotoxic T lymphocytes (CD8 +), "natural killer" T cells (NK), and macrophages (M) induce cholangiocyte apoptosis by releasing perforin, granzyme B, and soluble forms of FasL (sFasL), TRAIL (sTRAIL), and TNF-α (sTNF-α), or by binding the membrane-associated forms of these cytokines to their plasma mambrane receptors in cholangiocytes. CD8+ binding to bile ducts is facilitated by the expression in cholangiocytes of the *major histocompatibility complex* (MHC) class I antigen (MHC-I) and T cell adhesion molecules, such as the *intercellular adhesion molecules 1* (ICAM-1). NK bind to the cholangiocyte through the NKG2D ligand. Release of *danger-associated molecular patterns* (DAMPs) by death cholangiocytes activates the innate immunity, which involves DAMP recognition by *toll-like receptors* (TLRs) located in cholangiocytes, NK cells and granulocytes, and the subsequent release of inflammatory/proapoptotic mediators that induce apoptosis in intact cholangiocytes.

*pathogen-associated molecular patterns molecules* (PAMPs), which alert the immune system against microbial infections. DAMPs are nuclear and cytoplasmic molecules, including nucleosides, uric acid, and the proteins *chromatin-associated protein high-mobility group box 1* (HMGB-1), S100A8/S100A9 and heat shock proteins, among others [90]. Like PAMPs, DAMPs activate NK cells and granulocytes, and the further release of inflammatory/proapoptotic mediators, such as cytokines, chemokines, and reactive oxygen and nitrogen species, thus exacerbating the inflammatory response and contributing significantly to the severity of the cellular injury [91]. Human cholangiocytes express a variety of TLR isoforms, and at least TLR-2 and TLR-4 are functional to mediate the development of a pro-inflammatory phenotype after DAMP release [92]. B cells are hyper-responsive to innate stimuli, and therefore may contribute to the perpetuation of the autoimmune process [93].

In addition to stimulate the immunological cellular response, IL-2 released by CD4+ T cells also stimulates the humoral response, by triggering B-cell proliferation and immunoglobulin synthesis [94]. B cells produce IgG antibodies against either the haptenized proteins (anti-hapten antibodies) or the native ones (auto-antibodies), able to react against cholangiocyte-specific antigens expressed in the bile-duct surface. Opsonization of cholangiocytes may lead to direct attack by immune cells bearing IgG FCγ receptors against the FC moiety of these antibodies, such as NK cells, macrophages, and granulocytes, and to trigger complement-mediated cholangiocellular lysis [78,95].

All this severe inflammation accounts for cholangitis and cholangiolitis, which results in atrophy and progressive periductal fibrosis, bile duct degeneration followed by obstructive cholestasis and, eventually, loss of interlobular bile ducts [96]. This may even coexist with small bile duct proliferation, a phenomenon known as "ductular reaction," due to maturation of intermediate hepatobiliary cells originated from a proliferative behavior in the porto-hepatic interface [97].

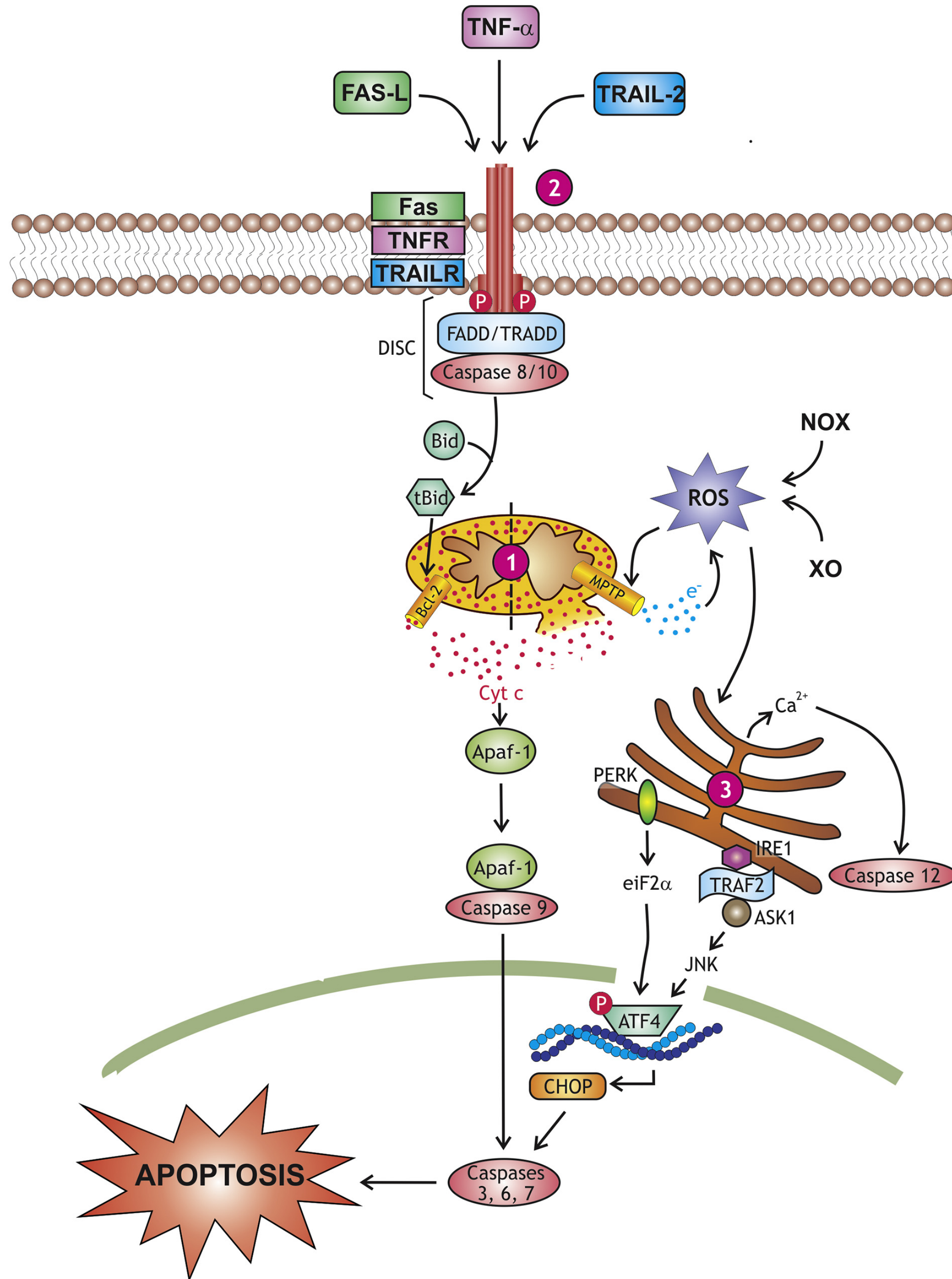
## 5. Mechanisms of cholangiocyte death

The mechanisms of cell death in cholangiocytes are common to the different ductopenic cholangiopathies. Cholangiocyte death is not as well characterized as hepatocyte death, but we know they comprise the three more important forms of cell death, namely apoptosis (type I), autophagy (type II), and necrosis (type III) [8,9].

The most common death mechanisms in biliary epithelial cells involved in ductopenic cholangiopathies are apoptosis and necrosis [63]. Autophagy and necroapoptosis has not been widely studied, but its role in ductopenic cholangiopathies is emerging [9].

### 5.1. Apoptosis

Apoptosis is the most widely studied death mechanism in

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cholangiopathies, and the main cause of ductopenia associated with them [98]. It results in characteristic morphological changes in cholangiocytes, including decreased cell size, chromatin condensation, and nuclear fragmentation; this leads to formation of apoptotic bodies, which are eliminated by phagocytosis of macrophages or neighboring epithelial cells [99,100]. Apoptosis is carried out through three pathways, namely extrinsic, intrinsic, and endoplasmic reticulum stress pathways (Fig. 4).

**Fig. 4.** Mechanisms of apoptosis in cholangiocytes: 1) Intrinsic or mitochondrial pathway of apoptosis, mediated by cytochrome *c* (Cyt *c*) release from the mitochondrial intermembrane space to the cytosol; Cyt *c* interacts with the adaptor protein *apoptotic protease activating factor-1* (APAF-1) to form the "apopsome" complex, which further recruits and activates procaspase-9 by proteolysis; once activated, caspase-9 activates the downstream executioners caspases 3, 6, and 7. Cyt *c* release is facilitated by a) the insertion in the outer mitochondrial membrane of pore-forming proapoptotic proteins of the Bcl-2 family (e.g. Bax, Bad), or b) the formation of mitochondrial permeability transition pores (MPTPs), which is triggered by reactive oxygen species (ROS) from different sources, including the pro-oxidative enzymes NADPH oxidasetrans (NOX) and xanthine oxidase (XO); the mitochondrial permeability transition also exacerbates electron leakage from the respiratory chain, thus promoting the generation of cytosolic ROS from mitochondrial origin. 2) Extrinsic pathway, initiated by a) binding of the pro-inflammatory cytokines *Fas ligand* (Fas-L), *tumor necrosis factor-α* (TNF-α), and *tumor necrosis factor-related apoptosis-inducing ligand-2* (TRAIL-2) to their respective plasma membrane receptors (Fas, TNFR, and TRAILR), b) autophosphorylation of these receptors, c) association of activated receptors with the death domains, *Fas-associating protein with death domain* (FADD) or *TNFR1-associated death domain protein* (TRADD), and with the procaspases 8 and 10 to form the DISC death complex, d) proteolytic activation of these caspases, and e) excision of Bid to truncated Bid (tBid), thus promoting formation of mitochondrial pores dependent of Bcl-2. 3) Endoplasmic reticulum (ER) stress apoptosis, which is induced by a ROS-mediated increase in $Ca^{2+}$ release and executed by caspase 12, or by the activation of the ER stress sensors *inositol-requiring transmembrane kinase/endoribonuclease 1* (IRE1) and *double-stranded RNA-dependent protein kinase* (PKR)*-like eukaryotic initiation factor 2α* (eIF2α) *kinase* (PERK). Both eIF2α, activated by PERK, and *cJUN NH2-terminal kinase* (JNK), activated by *apoptosis signal-regulating kinase 1* (ASK1) after IRE1-induced association of ASK1 with *TNFR-associated factor 2* (TRAF2), induce translocation of the transcription factor *eIF2α-activating transcription factor-4* (ATF4) to the nucleus, and the further expression of the proapoptotic transcription factor *C/EBP homologous protein* (CHOP). Regardless the pathway involved, endoplasmic reticulum stress-related apoptosis is the result of the downstream activation of the executioner caspases 3, 6, and 7 by caspase 12 and CHOP.

Both apoptosis pathways has been reported to be common mechanisms of cholangiocellular death in ductopenic cholangiopathies [29,101,102], either due to an autoimmune or infectious liver disease, or because of permanent exposure of the cholangiocytes to toxic drugs or endogenous damage by bile salts [8]. In the case of PBC, apoptosis of cholangiocytes of small ducts was shown to be secondary to the invasion of inflammatory cells [103]. As for PSC, a role for apoptosis in ductopenia is more controversial. Unlike what happens with PBC, staining for apoptosis was reported to be negative in liver samples of PSC patients [103]. However, further studies demonstrated that patients with PSC increases serum markers of apoptosis [104,105], and that they correlates with disease activity and prognosis [104].

#### 5.1.1. Extrinsic pathway

It is triggered by external activation of death receptors present in the plasma membrane, such as TNFR, Fas, and TRAILR. Activation of these receptors requires binding to their corresponding ligands, i.e., TNF-α, FasL, and TRAIL, all of which have cytokine activity [106]. The aforementioned ligands are transmembrane proteins from immune cells, and their soluble forms in plasma are due to the proteolytic cleavage of their extracellular domains by Zn-dependent metalloproteinases (for TNF-α and FasL) [107–109] or cysteine proteases (for TRAIL) [110]. Binding of any of these cytokines is required for apoptosis to take place. First, binding-dependent homo-oligomerization of the receptor is needed. Then, the *death-inducing signaling complex* (DISC) must be formed by association of Fas or TRAILR with the *TNFR-associated death domain* (TRADD) protein. Finally, DISC associates with procaspases 8 and 10 to activate them, and these active caspases activate, in turn, apoptosis executioner caspases, such as 3, 6, and 7 [106].

In the liver, the cytokines capable of activating the extrinsic pathway of apoptosis pathway are mainly produced by Kupffer cells. However, in bile ducts, the major sources of this type of cytokines are inflammatory lymphocytes and macrophages infiltrated in the biliary epithelium [111]. In addition, in several cholangiopathies, cholangiocytes themselves acquire a secretory phenotype associated with senescence, which aberrantly expresses pro-inflammatory receptors, chemokines, cytokines, and other growth factors that may sensitize surrounding cholangiocytes to cell death, as has been shown in PSC [111] and acute post-transplant cellular rejection [112]. Genetic and epigenetic factors may contribute to perpetuate this cholangiocellular phenotype, and the subsequent development of chronic effects, such as fibrosis, cholestasis, VBDS, and even cholangiocellular carcinoma [82].

Fas is a membrane receptor highly expressed in damaged cholangiocytes in PBC [98,113]. It is activated by its ligand, FasL, a cytokine expressed by macrophages and the cytotoxic T lymphocytes surrounding them or localized within the biliary epithelium, as as been shown in PBC [98]. Fas expression has been positively correlated with cholangiocyte apoptosis in PBC [114], with CD68+ monocytes surrounding the damaged bile ducts as a FasL source [115]. Its expression in PSC is much less pronounced, so its proapoptotic role could be considerably less relevant in this cholangiopathy [116].

The TNF-α/TNFR system is commonly involved in immunity-mediated ductopenia, by activating the extrinsic apoptosis pathway [117]. Moreover, cholangiocytes are the main source of TNF-α in the liver, and expression of TNF-α and its receptor are increased in several ductopenic cholangiopathies [118,119]. CD28+-inflammatory T cells located around the liver bile ducts of PSC patients are also an important source of TNF-α [120]. However, antibodies against TNF-α have been inefficient in patients with this pathology [121].

Regarding the TRAILR/TRAIL system, human cholangiocytes constitutively express TRAIL receptor type 2 (TRAILR2). In ductopenic cholangiopathies such as PSC and PBC, expression of this receptor is increased [28,119,122], an effect likely induced by the bile salts accumulated during the cholestatic process [123], via activation of the nuclear factor Sp1 by the *c-Jun N-terminal kinase* (JNK)-dependent pathway [124]. Serum levels of soluble TRAIL are also elevated in patients with PBC [119,125]. Systemic administration of TRAIL to mice induces a sclerosing cholangitis lesion, suggesting that endogenous TRAIL may contribute to human syndromes with these features, e.g., PSC [126,127]. Finally, in patients with BA, TRAIL levels are elevated, in association with an increase in activation of the pro-inflammatory transcription factor-κB (NF-κB) [6]. Nevertheless, none of the inflammatory cells that infiltrate the bile ducts in BA expressed other cytotoxic markers, such as perforin, granzyme B, and FasL [128].

#### 5.1.2. Intrinsic pathway

This apoptotic pathway is activated in response to a large number of cellular stress conditions, such as DNA damage, extracellular matrix detachment, hypoxia, loss of survival factors, and oxidative stress, among others. In this pathway, the mitochondrial internal membrane is permeabilized by formation of *mitochondrial permeability transition pores* (MPTPs), causing colloid-osmotic swelling of the mitochondrial matrix due to entry of small solutes; this causes rupture of the mitochondrial external membrane, release of cytochrome *c* into the cytosol, formation of the apoptosome along with caspase 9 and *apoptosis protease-activating factor-1* (APAF1) and, finally, the apoptosome-mediated activation of the executioner caspases 3, 6, and 7. The mitochondria can also become permeable by assembly into the external mitochondrial membrane of pore-forming proapoptotic proteins of the Bcl-2 family, such as Bax and Bak. These two proteins are also recruited to mitochondria by truncated Bid, originated from to proteolytic excision of a C-terminal fragment of Bid by the extrinsically activated caspases 8 and 10 [129]; this acts as a functional link between intrinsic and extrinsic apoptosis pathways. Nonetheless, these pro-apoptotic stimuli can be avoided by anti-apoptotic proteins of the same family, like Bcl-2, Bc-xL, and Mcl-1, since they sequester proapoptotic proteins of the Bcl-2 family, thus impairing their

membrane association and insertion, oligomerization, and pore formation [130]. Therefore, change of mitochondrial permeability depends ultimately on the balance between pro- and anti-apoptotic Bcl-2 families of proteins [131]. Loss or reduction of Bcl-2, Mcl-1, and Bcl-XL expression, thus promoting pore formation in the mitochondrial membrane by Bax/Bak, has been observed in small bile ducts upon rejection of a liver allograft [117], and in PBC [103,113,132,133]. In addition, since Bax is expressed throughout the biliary tree and Bcl-2 only in the interlobular bile ducts and bile ductules, susceptibility to pro-apoptotic stimuli is specially selective [134].

The perforin/granzyme B route is the most widely used by CD8+ cytotoxic T cells and NK cells to produce apoptosis in their target cells through the intrinsic pathway. These immune cells release granules containing these two proteins, and perforin forms pores in the plasma membrane of the target cell that enable the diffusion of granzyme B into the cytosol. Once there, granzyme B triggers apoptosis by activating executioner caspase 3, or by excising Bid, which leads to tBid formation and further activation of the intrinsic apoptosis pathway [79,135]. Finally, the immunological cells detaches from the apoptotic cell, and can further interact with another intact target cell [79].

Granzyme B-positive biliary cells are prominent in small bile ducts of PBC patients, and the transcriptional levels of perforin and granzyme B are elevated in liver tissue of these patients [136,137]. Caspase 3 activated by this pathway can potentially generate immunogenic fragments of PDC-E2, so it may contribute to the deposit of autoantigens and the production of antimitochondrial antibodies in this pathology [138].

Oxidative stress is a significant inducer of mitochondrial dysfunction, and as such, a relevant factor in the activation of the intrinsic apoptosis pathway. It has indeed been implicated in cholangiocyte apoptosis and necrosis secondary to immune aggressions, ischemia/ischemia-reperfusion, and exposure to toxic bile salts [139]. Oxidative stress induces drastic changes in the permeability of the plasma membrane, release of mitochondrial components to the cytosol, decreased production of mitochondrial adenosine triphosphate (ATP), and, ultimately, apoptosis or necrosis, depending on the number of mitochondria affected; since apoptosis but not necrosis is an energy-dependent process, the latter predominates when ATP is depleted [140]. Oxidative stress has been shown to be independently associated with more advanced stages of some cholangiopathies as for example PBC [141]. Furthermore, expression of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was preferentially detected in the nuclei of damaged interlobular bile ducts in this cholangiopathy [142,143]. In response to DNA damage, the gene encoding the cyclin-dependent kinase inhibitor WAF1 (aka p21) is induced by upregulated p53; WAF1 is a potent and reversible inhibitor of cell cycle progression at both the G1 and G2 checkpoints, and upregulated WAF1 induces irreversible G1 arrest and apoptosis. This reactive oxygen species (ROS)-mediated genotoxic mechanism has been shown to be a causal factor of cholangiocyte apoptosis in PBC [144]. In PSC, ductular lesions seem to be secondary to the production of ROS by leukocyte metabolic activation induced by antibodies against the cytoplasm of neutrophils, which is found in the serum of more than 50% of PSC patients [145].

The origin of oxidative stress in cholangiopathies is multifactorial. Once activated, lymphocytes generate an increase in ROS, and this phenomenon is involved in the apoptosis occurring in ductopenic diseases, e.g., in liver allograft rejection [139]. Mitochondrial dysfunction is another source of ROS. Cytochrome *c* release from damaged mitochondria affects the electron flow in the respiratory chain, thus inducing *i*) over-reduction of mitochondrial complexes and electron leakage into the cytosol, with the subsequent formation of ROS, and *ii*) decrease in the electron acceptor $NAD^+$, which results in ROS emission from the α-ketoglutarate dehydrogenase complex [146]. Oxidative stress can also be produced by enzymes that produce ROS as a consequence of their catalytic activity, such as NADPH oxidase (NOX) and xanthine oxidase (XO). NOX is a membrane-integrated enzyme that transports electrons through the plasma membrane and generates superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$) from molecular oxygen inside the cells, by using NADPH as an electron donor [147]. XO, instead, produces $H_2O_2$ by catalyzing the oxidation of hypoxanthine to xanthine, and its subsequent oxidation to uric acid [148]. Both pro-oxidative enzymes are involved in ischemic damage to the bile ducts, e.g., in hepatic arterial stenosis, after liver transplantation, or in common bile duct compression after infusion of intra-arterial chemotherapeutic drugs [149]. Finally, the infiltrated eosinophils can be another source of ROS in PBC, since expression of eosinophil peroxidase, an eosinophilic granule protein that produces the powerful oxidant hypochlorous acid (HOCl), is increased within the portal space of PBC patients; macrophages also show evidence of the phagocytized enzyme [150]. The high sensitivity of cholangiocytes to oxidative stress has been attributed to their low GSH content, which is only 1/3 of that of hepatocytes [151], and cholangiocyte GSH levels are further decreased in cholangiopathies, such as PBC [150].

#### *5.1.3. Endoplasmic reticulum stress*

This proapoptotic pathway is triggered when the production and transport systems of proteins are altered, leading to accumulation of misfolded proteins, and eventually, apoptosis. Endoplasmic reticulum stress is triggered by several factors, such as oxidative stress, $Ca^{2+}$ accumulation in the organelle, nutrient deprivation, and a large number of toxic aggressions, among others [152].

Endoplasmic reticulum stress is also triggered by loss of $Ca^{2+}$ homeostasis due to its excessive release of the cation from this organelle; under this condition, $Ca^{2+}$-dependent calpains activate caspase 12, which in turn activates the executioner caspases 3, 6, and 7 [153,154]. Similarly to what happens in mitochondria under pro-apoptotic conditions, Bax and Bak can migrate to the endoplasmic reticulum, where they regulate $Ca^{2+}$ channels and the inositol 1,4,5-trisphosphate receptor, thus potentiating $Ca^{2+}$-dependent apoptosis. Furthermore, protein misfolding can activate c-Jun through the JNK-dependent proapoptotic route, and induce expression of proapoptotic transcription factor CHOP, which favors apoptosis by modulating activity and expression of members of the Bcl-2 protein family [155].

Although there is no direct evidence of the involvement of endoplasmic reticulum stress as an apoptosis mechanism in ductopenic cholangiopathies, the machinery enabling this pathomechanism is completely functional in cholangiocytes. Furthermore, it can be activated by the high levels of cytotoxic, endogenous bile salts at which they are exposed in obstructive cholangiopathies [35]. Moreover, expression of PDI and GRP78, two endoplasmic reticulum stress markers, was significantly higher in small bile ducts, which are the most affected ones in PBC [9,35].

A possible mechanism accounting for the oxidative and endoplasmic reticulum stress in PBC is the microRNA (miR) dysregulation that occurs in hepatocytes and in immune cells of these patients. miR-506 is overexpressed in cholangiocytes of PBC patients, through a mechanism involving proinflammatory cytokines that are usually found to be elevated in PBC livers, such as IL-8, IL-12, IL-17, IL-18, and TNF-α [156]. Experimental overexpression of miR-506 in cholangiocytes affected mitochondrial energetic metabolism, with increased oxygen consumption, glycolysis, and proton leakage; this indicates uncoupling of respiration, which may have in turn accounted for by the increase in oxidative and endoplasmic reticulum stress [156]. Overexpression of miR-506 also sensitized cholangiocytes to bile acid-induced apoptosis, via a caspase 3-dependent mechanism [156]. Impairment of the $HCO_3^-$ umbrella is a likely mechanism explaining the increased vulnerability to bile acid-induced apoptosis, since overexpression of miR-506 also decreases the $Cl^-/HCO_3^-$ exchanger AE2 expression in cholangiocytes from PBC patients [157], and impaires type III inositol 1,4,5-trisphosphate receptor [158]; this is the major isoform of the receptor in cholangiocytes, where it regulates $HCO_3^-$ and $Cl^-$ secretion into bile [159].

### 5.2. Autophagy

Autophagy is regarded as a cellular protective process that eliminates damaged or aged proteins and organelles [9]. It also play a protective role during acute cellular stress (e.g., nutrient deprivation), thus helping surrounding cells to maintain cellular energy homeostasis [35]. Nevertheless, under chronic stress conditions, it represents an alternative mechanism of cell death to apoptosis; actually, these two self-destructive processes share common executer pathways, and are functionally linked [63,160,161]. The first step of autophagy is the nucleation of a membrane of unknown origin, although it seems to come from the endoplasmic reticulum, the Golgi apparatus, or *de novo* synthesis. This initial membrane, referred to as "phagophore", is elongated until the edges are fused, thus producing a double-membrane vacuole, the "autophagosome", that encapsulates the material to be degraded. Once the autophagosome is formed, it fuses with the lysosome, thus creating the autophagolysosome; lysosomal enzymes degrade its content, which allows for the degradation of aged or damaged cell material [162].

There is accumulating evidence that autophagy plays a major role in PBC pathogenesis. Cytoplasmic vesicles containing the autophagy-related marker *microtubule-associated protein-light chain 3* (LC3) has been usually found in injured bile ducts in this hepatopathy [35]. Autophagy seems to be related to the autoimmune process against mitochondrial antigens, by regulating T lymphocytes [63]. In a study where cholangiocytes in culture were exposed to several forms of stress, cells showed LC3 and PDC-E2 co-localization followed by PDC-E2 expression on the cell surface, suggesting that autophagy is responsible for granular expression and subsequent cell surface expression of PDC-E2 [160].

Autophagy may also precede biliary epithelial senescence in cholangiopathies, a process that seems to be involved in bile duct lesions [163]; almost 90% of hepatobiliary cells express senescence markers in cholestatic cholangiopathies [164]. Cholangiocyte senescence can be triggered by different cellular stresses occurring in these hepatopathies, such as oxidative stress and DNA damage [165]. Senescence is a state of irreversible growth arrest in the G1 phase of the cell cycle [166], and therefore, accumulation of senescent cholangiocytes may contribute to loss of functional biliary mass [164]. Furthermore, senescent cholangiocytes express various cytokines and chemokines that may exacerbate the inflammatory microenvironment around bile ducts, thus contributing to pathogenesis [163]. Cholangiocyte senescence during cholestatic liver injury has been linked to the epigenetic regulation of the expression of the positive regulator of biliary development FoxA2, as has been shown for PBC, PSC, and BA [164,167]; the proto-oncogene N-Ras, a known inducer of senescence, has been also causally linked to senescence in PSC cholangiocytes [111].

### 5.3. Necrosis

Generally, necrosis is a consequence of acute and severe metabolic perturbations, such as those that take place in ischemic cholangiopathies or drug-induced toxicity. This leads to an abrupt increase in the plasma membrane permeability and, eventually, cell lysis. As a consequence, a strong inflammatory response is triggered due to the massive release of DAMPs to the extracellular medium, which activates the innate immune system and initiates a secondary cascade of damage and inflammation against bile ducts [82,89,160].

Necrosis is the main cell death mechanism when there is massive formation of MPTPs, leading to a significant decrease in cellular ATP levels [168,169]. This type of ductopenic lesion predominantly affects the middle third of the bile ducts, leading to hepatic duct confluence, with intrahepatic affectation rarely being present [170]. In PBC, focal lesions display severe inflammatory changes and necrosis around bile ducts, often referred to as "florid duct lesion" [171]. The lymphocytes and mononuclear cells that constitute the inflammatory infiltrate are in close contact with the basal membrane of cholangiocytes undergoing necrosis [171]. In addition, non-anastomotic biliary strictures and bile duct necrosis have been described as frequent complications in liver transplantation related to ischemia-reperfusion injuries [172]. A role for necrosis in PSC ductopenic lesions is doubtful, however, since biochemical markers of necrosis are not elevated in PSC patients [105].

### 5.4. Necroptosis

Necroptosis, a controlled and genetically regulated form of necrosis that resemble apoptosis regarding its "programmable" trait [173], also occurs in cholangiopathies [174,175]. Necroptosis is similar to necrosis in terms of the kind of cellular alterations observed, as it shows plasma membrane permeation, colloid osmotic swelling (oncosis), mitochondrial dysfunction, and release into the extracellular space of the cytoplasmic content, also inducing inflammatory reactions due to DAMP release [173].

It is induced by similar factors to necrosis (e.g., intracellular ATP depletion, loss of $Ca^{2+}$ homeostasis, mitochondrial depolarization, proteolysis by non-apoptotic proteases, and ROS increase), but it differs in that it can be induced, like apoptosis, by activation of cell surface receptors, mainly TNFR1, although Fas and TRAILR can be involved as well [176]. Caspase 8, the initiator of this process, is normally involved in apoptosis and not necrosis, because it simultaneously inhibits the excision of *receptor-interacting serine/threonine-protein kinase* (RIP)-1 and 3, two molecules that execute necrosis [177]. However, under certain conditions where caspase 8 is inhibited, RIP1 and RIP3 form the "necrosome" with other proteins, and cause necrosis through the *mixed lineage kinase domain-like* (MLKL)-mediated mitochondrial pathway, which involves the recruitment of the mitochondrial protein phosphatases PGAM5L and PGAM5S, and the further *dynamin-related protein-1* (Drp1) activation; this latter protein triggers mitochondrial fission and ROS production, thus causing a cellular damage similar to that observed in necrosis [178].

High expression levels of RIP3 and MLKL have been detected in the liver of PBC patients [175]. Moreover, mice with a RIP3 genetic ablation subjected to obstructive cholestasis by bile duct ligation showed decreased oxidative stress levels, inflammation, and necrosis, suggesting a role of this cell death mechanism in obstructive cholangiopathies [175]. A role for the microRNA miR-21 in cholangiocyte necroptosis has been suggested by studies showing that it is particularly overexpressed in biliary cells in human, as well as in both bile-duct ligated and Mdr2 knockout mice [179]. Finally, although studies of liver transplantation are lacking, necroptosis may play a role in ischemia-reperfusion injuries and also in liver allograft rejection, as inferred from the occurrence of similar injuries after renal transplantation [180].

## 6. Therapeutic approaches to limit ductopenic injury in cholangiopathies

Since there is a lack of therapeutic strategies to stimulate cholangiocellular proliferation in order to replenish bile duct under ductopenic conditions, current therapeutic strategies are limited to protect cholangiocytes against cell death mechanisms operating via either the exacerbated immunological response or exposure to luminal factors associated with the occurrence of a "toxic bile" in cholangiopathies. The current therapeutic strategies to counteract cholangiocyte death include *i*) the anticholestatic drug ursodesoxycholic acid (UDCA), which may protect these cells from apoptotic and possibly necrotic death induced by deleterious bile salts accumulated during the obstructive process and by cytokines released during the inflammatory process, and *ii*) immunosuppressive and anti-inflammatory agents, when ductopenia is suspected to be predominantly associated with immune-mediated mechanisms.

### 6.1. UDCA

This hydrophilic, non-toxic bile salt is used nowadays as the first-choice drug for the treatment of cholestasis in general, and for most cholangiopathies in particular [181]. For example, UDCA has been approved by the Food and Drug Administration for the treatment of PBC [171]. Moderate UDCA doses has been recommended by the European Association for the Study of the Liver for PSC treatment based upon results showing improvement in liver function tests and surrogate markers of prognosis in these patients, although no improved survival was demonstrated so far [21]. As for CF-associated liver disease, even when there is no established therapy for this cholangiopathy, UDCA therapy is highly recommended, since it was suggested to reduce bile viscosity [182,183]. UDCA could be also useful as an adjuvant therapy in BA, where biochemical benefit has been shown in a single crossover trial in older children with the disease after successful surgery [184]. Finally, UDCA could be beneficial even under conditions of complete extrahepatic obstruction, at least when administered at low doses and during a short administration period [185].

A compelling body of experimental and clinical evidences points UDCA as a bile duct-protective agent due to its well recognized capability to counteract bile salt-induced apoptosis and necrosis, and due to its alleged immunosuppressive and immunomodulatory properties.

Its protective mechanisms are as follows:

1) UDCA displaces and replaces highly toxic, endogenous bile salts accumulated in cholestatic hepatopathies; UDCA comprises no more than 4% of the total endogenous bile acid pool, but its value is increased to 40–60% under a conventional UDCA therapy [186]. Since biliary bile salt composition reflects the plasmatic one, the bile salt biliary would be far more harmless to cholangiocytes in patients on UDCA.
2) UDCA may counteract hepatic bile acid-induced cell death in liver. Although there is no direct evidence in cholangiocytes, UDCA have well-documented anti-apoptotic [187] and anti-necrotic [188] properties both in hepatocytes and in other extrahepatic cell types. UDCA has anti-apoptotic effects secondary to mitochondrial injury by blocking MPTP formation induced by both bile salts [189,190] and proinflammatory cytokines, including TNF-α [191], FasL [192], and transforming growth factor-β1 (TGF-β1) [193]. UDCA also reduces the expression of the members of the Bcl-2 family of proteins that form mitochondrial pores (e.g., Bad and Bax) by inhibiting p53, a pro-apoptotic transcription factor that induces Bad and Bax expressions [194]. UDCA also counteracts *t*Bid-induced the mitochondrial pore formation [192], and the upregulation of AP-1, a pro-apoptotic transcription factor activated via the TNFR/TNF-α signaling pathway [195]. Finally, tauroursodeoxycholic acid (TUDCA), a main UDCA metabolite, inhibits endoplasmic reticulum stress-induced apoptosis both by inhibiting caspase-12 activation via modulation of intracellular $Ca^{2+}$ levels [196], and by acting as a chemical chaperone that counteracts endoplasmic reticulum stress itself [197]. Apart from its anti-apoptotic effects, UDCA can activate survival pathways. TUDCA binds to the *epidermal growth factor receptor* (EGFR), which activates the signaling survival pathways mediated by *extracellular signal-regulated kinase* (ERK) and *phosphoinositide 3-kinase* (PI3K)-Akt [198,199]. This mechanism of UDCA protection was confirmed in a rat model of apoptosis-induced ductopenia by combined vagotomy and bile-duct ligation [200].
3) UDCA reinforces adaptive mechanisms that the liver evokes spontaneously in cholestasis to attenuate the damaging effects of accumulated bile salt on hepatocytes and cholangiocytes. This adaptive response involves *i*) decrease of intracellular (and hence intrabiliary) levels of bile salts by repression of Cyp7a1, the rate-limiting enzyme of bile salt synthesis [201]; *ii*) attenuation of bile salt toxicity by promoting formation of less harmful polyhydroxylated bile salts, via induction of sterol hydroxylases [202]; *iii*) downregulation of sinusoidal bile-salt uptake transporters, e.g., *$Na^+$-taurocholate cotransporting polypeptide* (Ntcp), and upregulation of basolateral bile salt extrusion pumps systems, e.g., *multidrug resistant-associated protein* (Mrp) 3 and 4, which prevents bile salts from building up in liver by diverting them to urine. Patients on UDCA might activate some of these adaptive mechanisms in part by transactivating the nuclear receptor farsenoid X receptor (FXR) [202], but this response can be reinforced by the concomitant administration of the more potent FXR ligand obethicholic acid [203], or by supplementation with rifampicin, which activates pregnane X receptor (PXR), another nuclear receptor with complementary beneficial mechanisms to FXR [204].
4) UDCA restores ductular mechanisms of defense against toxic bile salts, which are frequently altered in ductopenic cholangiopathies [7]. This is accounted for by *i*) stimulation of MDR3-mediated phospholipid biliary excretion [204], an effect that can be reinforced by peroxisome proliferator activated receptor-α (PPAR-α) ligands, such as fibrate drugs [205], and *ii*) stimulation of $HCO_3^-$ ductular excretion, with the concomitant reinforcement of both the "bicarbonate umbrella" and the hypercholeresis-induced dilution of toxic luminal bile salts; this occurs (*1*) by the stimulation of cholangiocyte $HCO_3^-$ secretion via AE2 by transcriptional and post-ranscriptional mechanisms [181,206], particularly when combined with glucocorticoids [207], and (*2*) by the ability of unconjugated UDCA to generate luminal $HCO_3^-$ molecules as part of its characteristic cholehepatic recirculation. The UDCA homologue *nor*-UDCA, which suffers cholehepatic shunting even more efficiently than UDCA and has a higher osmotic choleretic efficiency [208], bore better therapeutic effects than UDCA to ameliorate sclerosing cholangitis in Mdr2-knockout mice, which have a complete inability to secrete phospholipid into bile [209]. Furthermore, *nor*UDCA has promising anticholestatic effects in patients with PSC, according to a recent phase II clinical study [210].
5) UDCA bears anti-inflammatory and immunomodulatory properties, in part due to its capability to bind and further activate the glucocorticoid receptor [211]. UDCA may inhibit humoral autoimmunity, as suggested by its ability to inhibit IgM, IgG, and IgA production by B cells exposed to bacteria [212]. UDCA also counteracts the cellular immune response by inhibiting the release of cytokines produced by mononuclear cells, such as IFN-α, IL-2, and IL-4 [212], a finding that was confirmed for IL-2 in a cholangitis experimental model [213]. UDCA may also counteract the overexpression of MHC-II [214] and ICAM-1 [215] in the apical membrane of the biliary epithelial cells, as well as of the ICAM-1 partner receptor in lymphocytes, *lymphocyte function-associated antigen 1* (LFA-1) [215].

### 6.2. Immunosuppressant agents

Despite many cholangiopathies are considered autoimmune diseases, they not usually respond to conventional immunosuppressive drugs (e.g. azathioprine, chlorambucil, cyclosporine, methotrexate, mycophenolate mofetil), including PBC [216,217] and PSC [218]. These drugs were either only marginally effective, ineffective, or even detrimental, and this is why they are not currently recommended to treat these liver diseases [219]. Similarly, the treatment of PBC with the corticosteroid budesonide is controversial, and should be reserved to non-cirrhotic patients with PBC/autoimmune hepatitis overlap syndrome [21]. A 1-year pilot trial in PBC patients treated with prednisolone showed some improvement in histology and liver function tests, but also a marked increment in bone loss [220]. Finally, a further study combining prednisolone with UDCA showed histological recovery at early PBC stages, but was not superior to UDCA monotherapy [216].

Corticosteroids have been also regarded as mostly ineffective in drug-induced VBDS by some specialists [14]. However, the often association of VBDS with drug-induced hypersensitivity reactions justify the pharmacological induction of immunosuppression as part of the

general therapeutic strategy for this particular case. Benefits have been supported by anecdotal cases of drug-induced ductopenia in Stevens-Johnson syndrome [221–223], toxic epidermal necrolysis [224–226], erythema multiforme [227], and hemophagocytic lymphohistiocytosis [228]. Corticosteroids are the most common immunosuppresors used in these cases, either alone or together with other immunosuppressive drugs when the patient is unresponsive to corticosteroids. Alternative immunosuppressants applied with some success include mycophenolate moefetil [229], tacrolimus [230], and cyclosporine [225]. Other therapeutic strategies for immunologically mediated diseases, such as administration of infliximab (a monoclonal antibody against human TNFα) and plasmapheresis, have been also successfully employed in these particular cases [224].

### 6.3. Stimulators of cholangiocyte proliferation

There is no current established therapy to treat ductopenia based upon stimulation of cholangiocyte proliferation.

Cholangiocyte proliferation occurs spontaneously in early stage of the cholangiopathy course as a consequence of the inflammatory process, and the mechanisms involved in this adaptive response may be useful to envisage therapeutic strategies based upon its stimulation. This phenomenon, referred to as "ductular reaction", consist of the formation of small epithelial tubules, with cholangiocytes been produced from hepatic progenitor cells within the Canal of Hering that are activated during liver injury [231,232]. Peribiliary glands that are lined with biliary epithelial cells of large intrahepatic bile ducts and extrahepatic bile duct are also a source of multipotent stem/progenitor cells for cholangiocyte proliferation and renewal [233,234].

Regulatory aspects of this proliferative process have been intensely studied in animal models of biliary hyperplasia, such as bile duct ligation (BDL), partial hepatectomy, chronic bile acid feeding, and more relevant to our issue, in the ductopenic model of administration of $CCl_4$ to bile-duct ligated rats [71,235,236]. Using these models, several signaling pathways have been identified to regulate proliferation and migration of resident cells in cholangiocyte progenitor niches. After BDL, large cholangiocytes lining larger bile ducts, but not small cholangiocytes lining smaller bile ducts, proliferate through the cAMP-mediated activation of the PKA-Src-MEK-ERK1/2 signaling pathway stimulated by secretin [235,237]; the reason for this difference is that small cholangiocytes do not express secretin receptor, and are normally mitotically dormant [238,239]. However, small cholangiocytes can proliferate via activation of the *$Ca^{2+}$/calmodulin/calmodulin-dependent protein kinaseI* (CaMKI)/*cAMP-response element binding* (CREB)-dependent signaling pathway by certain stimuli, such as H1 histamine receptor stimulation [240]. In pathologic conditions of large cholangiocyte damage (e.g., after $CCl_4$ or γ-aminobutyric acid treatment), small cholangiocytes replenish the damaged biliary tree by both amplification of this $Ca^{2+}$-dependent signaling pathway and transdifferentiation to the large cholangiocyte phenotype [241,242]. These and other hyperproliferative signaling pathway (e.g., the PKCβ-I-dependent one) are evoked by different signaling modulators with potential to be used as coadjuvant agents for ductopenia treatment, including neurotransmitters/neuromodulators (e.g., dopamine, neural growth factor, acetylcholine, epinephrine, calcitonin gene-related peptide, acetylcholine, norepinephrine, anandamide, and histamine) and hormones (e.g., growth hormone, vascular endothelial growth factor, epinephrine, insulin-like growth factor-1, prolactin, melatonin, angiotensin, glucagon-like peptide-1, and different sex hormones) [235,243], acting either from blood and through innervations by the autonomic nervous system, respectively. Alternatively, autocrine/paracrine mechanisms have also been described, which are associated with an increased transdifferentiation of proliferating small cholangiocytes towards a neuroendocrine phenotype able to secrete many of these mediators, and bind them via expression of their receptors [244]. This greater plasticity and proliferative potential of small cholangiocytes as compared to more differentiated, large ones points the former as a functional hepatobiliary progenitor cell population that can be therapeutically targeted to replenish the biliary epithelium in ductopenia. Whatever is its nature, this treatment should be applied early during the ductopenic process, since bile ducts are only rarely reconstructed as complete epithelium-lined tubes once they have been fully destroyed [245].

Caution is warranted when designing these therapeutic strategies, due to putative adverse effects associated with exacerbated cholangiocyte proliferation. First, proliferating cholangiocytes secrete a number of mediators, such as cytokines, chemokines, and other profibrogenic factors, that act via paracrine mechanisms to stimulate myofibroblast activation, migration, and proliferation, thus promoting liver fibrosis [96]. Second, the stimuli needed to activate otherwise quiescent cholangiocytes may trigger uncontrolled cholangiocyte hyperproliferation in susceptible individuals, resulting in liver diseases associated with growth-promoting effects, such as polycystic liver diseases [246] and cholangiocarcinoma [247]. This therapeutic approach may be particularly dangerous in diseases with a high lifetime risk of developing cholangiocarcinoma, such as PSC and CF-associated liver disease, especially if associated with an adjuvant antiapoptotic therapeutic strategy [248]. The crucial question here is whether we will be able to specifically stimulate those pathways that are beneficial for bile duct reconstitution without activating those causing harmful effects, or alternatively, to antagonize the unwanted effects without affecting the beneficial ones. The better comprehension of the signaling pathways that regulate differentiation and proliferation of both stem/progenitor cells and mature cells is clearly needed to develop such novel regenerative therapies.

## 7. Conclusions

The important advances in understanding cell death mechanisms in general, and the search for evidence of their particular involvement in cholangiopathies, have allowed us to understand in more detail the nature of these liver diseases. This knowledge has allowed us to explain why, under certain conditions or in certain individuals, the cholangiocellular death rate exceeds the cell proliferation rate, thus leading to different degrees of ductopenia. In addition to clearing the main functional cells out of the bile ducts causing both functional and obliterative cholestasis, the exposure of immunological cells to intracellular, potentially antigenic components released by death cholangiocytes is a powerful inflammatory stimulus that perpetuates a vicious cycle by which inflammation leads to cholangiocyte death, which in turn induces even more inflammation. Therefore, to limit this process is critical to improve disease prognosis.

The studies conducted in this field have allowed us to establish that ductopenic disruption is closely related to cholangiocellular death mechanisms, with apoptosis being the major death pathway involved, and the one we know currently in greater detail. However, our knowledge of other less studied cell death mechanisms to which cholangiocytes might be exposed in the context of each cholangiopathy, as well as their mechanisms of initiation and regulation, is still largely insufficient. In this sense, more studies on the role of autophagy and necroptosis, two recently acknowledged cholangiocellular mechanisms of cell death in ductopenic cholangiopathies, are eagerly awaited to better understand their role in the physiopathology of the disease. But at the same time, we must understand more thoroughly the defense mechanisms of the cholangiocyte that allow it to survive, in order to modulate them pharmacologically. Finally, making progress that allows us to envisage repair mechanisms against the damage suffered in each hepatopathy is crucial. Unfortunately, there are no well established therapeutic strategies to stimulate cholangiocellular proliferation in order to counteract ductopenic conditions through bile duct replenishment that lack risks associated with excessive growth-promoting effects. Meanwhile, even more efforts to envisage new modes to counteract the exacerbated cell death must be made, in order to restore

the balance between cell death and proliferation, by simultaneously improving both sides of the equation. This challenge will also involve answers to the question on how gradients in cholangiocyte phenotypes along the biliary tree will be reconstituted to assure an appropriate ductal structure and function at the organ level.

## Conflicts of interest

All authors declare no conflict of interest.

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