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Role of extracellular vesicles in hypoxia-induced hepatic injury in non-alcoholic fatty liver disease

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DOI:
[10.33612/diss.180853744](https://doi.org/10.33612/diss.180853744)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Hernandez Villanueva, A. (2021). *Role of extracellular vesicles in hypoxia-induced hepatic injury in non-alcoholic fatty liver disease*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.180853744>

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Role of extracellular vesicles in hypoxia- induced hepatic injury in non-alcoholic fatty liver disease

PhD Thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on
Wednesday 6 October 2021 at 14.30 hours

by

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FACULTAD DE MEDICINA
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ROLE OF EXTRACELLULAR VESICLES IN HYPOXIA-INDUCED HEPATIC INJURY IN NON- ALCOHOLIC FATTY LIVER DISEASE

TESIS PARA OPTAR AL GRADO DE DOCTOR
EN CIENCIAS MÉDICAS EN COTUTELA CON LA
UNIVERSIDAD DE GRONINGEN

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To my parents Victor and Gloria for pushing me to spread my wings and for always trusting me,

To my lovely family, brothers, nephews and aunts for their unconditional support,

To my soul friends who are my other family that I chose,

To my tutor, specially Han and Marco, that I owe every education and professional training,

To my boyfriend Pedro for being the happiness of my life,

To each one of you ... I always carry in my heart.

A mis padres por impulsarme a abrir mis alas y por confiar siempre en mí,

A toda mi familia por su amor incondicional,

A mis amigas del alma que son mi otra familia que elegí,

A mis profesores que les debo cada enseñanza y formación profesional,

A mi pololo por ser la alegría de mi vida,

A cada uno de ustedes...los quiero y llevo siempre en mi corazón.

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Preface & Scope of this doctoral thesis

PREFACE

Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent chronic liver disease that affects 30% of the general population. The incidence of NAFLD is still increasing. NAFLD encompasses a pathological spectrum of liver injury, ranging from isolated steatosis to an inflammatory condition termed non-alcoholic steatohepatitis (NASH), that can progress to liver fibrosis and subsequent cirrhosis and hepatocellular carcinoma (HCC). Clinical observations have indicated that obstructive sleep apnea syndrome (OSAS) is a significant risk factor that predisposes patients to the progression of liver steatosis, inflammation and fibrosis. OSAS is a sleep breathing disorder characterized by intermittent hypoxia (IH) during sleep. It has been shown that IH in patients and in experimental rodent models is linked to oxidative stress, steatosis, inflammation and liver fibrosis.

In recent years, extracellular vesicles (EV) have been implicated in intercellular communication in various pathophysiological conditions, including NASH. However, the mechanisms underlying the role of EV in NASH in the context of (intermittent) hypoxia remains unexplored.

This doctoral thesis focuses on the role of extracellular vesicles in the pathogenesis of hypoxia-induced hepatic injury in different models of NASH. To validate our hypothesis we used *in vitro* models to investigate cellular crosstalk between fat-laden hepatocytes exposed to chemical hypoxia and non-parenchymal cells, such as hepatic stellate cells and Kupffer cells. Also, we used an *in vivo* model of NASH to evaluate whether IH promotes liver injury and increases circulating EV. Our findings reveal new insights on the pathophysiological effects of hypoxia on lipotoxicity, inflammation and fibrosis. Moreover, we provide novel insights with regard to the presence of caspase-1 in EV, suggesting caspase-1 as a potential novel biomarker to monitor NAFLD and OSA.

SCOPE OF THE THESIS

The research described in this thesis focuses on the combination of lipotoxicity and hypoxia in *in vitro* and *in vivo* models of NASH. We investigate whether hypoxia promotes the activation of inflammatory and fibrotic pathways, with a special emphasis on the role of EV in inflammasome activation.

In Chapter 1 we present a general introduction of NAFLD, intermittent hypoxia-related OSA, the role of EV in NAFLD/NASH and the general aims of this thesis.

In Chapter 2, the role of EV in liver pathophysiology is reviewed and discussed, including their potential applications as biomarkers and therapeutics.

In Chapter 3 we introduce our experimental *in vitro* model of NASH using primary rat hepatocytes exposed to free fatty acids (FFA) and subjected to chemically-induced hypoxia (CH) using the hypoxia-inducible factor 1 alpha (HIF-1 α) stabilizer cobalt chloride (CoCl₂). We observed that hypoxia aggravates hepatocellular injury via a mechanism that involves inflammasome/caspase-1 activation in fat-laden hepatocytes and cellular crosstalk with Kupffer cells through EV. Furthermore, we

In Chapter 4, we study the cellular crosstalk between hypoxic human HepG2 cells exposed to FFA and human hepatic LX-2 stellate cells. We observed that hypoxia exacerbates hepatocellular damage and pro-fibrotic signaling and that this is correlated with increased EV release from fat-laden HepG2 cells. Interestingly, EV from hypoxic fat-laden HepG2 evoked a pro-fibrotic response in LX-2 cells.

In Chapter 5 we show an important finding about caspase-1 as one of the cargo components of EV from both hypoxic fat-laden hepatocytes as well as from serum of mice fed the CDAA diet and exposed to IH. Moreover, we observed that silencing of HIF-1 α in hepatocytes abolished the induction of inflammasome-related genes in Kupffer cells. These data suggest that IH could be an aggravating factor in the progression of NASH via HIF-1 α induction

Finally, in Chapter 6 we summarize and discuss our results and provide an outlook for future studies.

Chapter 1

1. - GENERAL INTRODUCTION AND AIMS OF THE DOCTORAL THESIS

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide, affecting up to 30% of the current population (1). NAFLD is used as an umbrella term that describes a clinico-pathological entity defined by the presence of a spectrum of hepatic histological changes. Observed phenotypes vary in severity from non-inflammatory isolated steatosis (also termed non-alcoholic fatty liver [NAFL]) to a more aggressive form named steatohepatitis (or non-alcoholic steatohepatitis, NASH), which is characterized by inflammatory changes and hepatocellular ballooning associated with varying degrees of liver fibrosis (2,3). NASH is a multisystem disease associated with a significant risk for the development of cirrhosis and hepatocellular carcinoma, as well as liver transplantation and liver-related death (4,5).

The pathogenesis of NAFLD involves a complex interaction between nutritional factors, obesity, insulin resistance, changes in the microbiota, genetic and epigenetic factors. These factors are involved in the development and progression of steatosis resulting in an excessive accumulation of lipids, especially triglycerides, in the hepatocytes (6-8). Moreover, these factors contribute, either simultaneously or sequentially, to the inflammatory and fibrotic response of the liver to steatosis (9). At the cellular level, the increased influx of free fatty acids (FFA) into the liver exceeds the physiological capacity, leading to reactive oxygen species (ROS) overproduction, mitochondrial dysfunction and cellular death in a process called lipotoxicity, ultimately leading to inflammation and fibrosis (10,11).

Several studies have shown that lipotoxicity appears to be the central driver of hepatocellular damage that contributes to the inflammatory response and the development of liver fibrosis (12,13). The crosstalk between hepatocytes and non-parenchymal cells plays a crucial role in this inflammatory and fibrotic response (14-17). It is believed that fat-laden (steatotic) hepatocytes release damage signals to the extracellular environment, resulting in paracrine effects on neighboring cells such as resident macrophages of the liver (Kupffer cells) and stellate cells that promote the activation of inflammatory and fibrogenic pathways, respectively (18,19). In addition, fat-laden hepatocytes trigger various pathways leading to hepatocellular dysfunction by autocrine effects that initiate and perpetuate lipotoxicity (20-22).

In recent years, obstructive sleep apnea syndrome (OSAS), a common sleep disorder characterized by recurrent closure of the upper airways during sleep, has been associated with the development and progression of NAFLD (23) in both obese and non-obese subjects (24-25). The most noted hallmark of OSAS is intermittent hypoxia (IH), which leads to tissue hypoxia and can result in ROS

overproduction, mitochondrial dysfunction and inflammation (26). These events are also involved in the initiation of NASH (27). Several studies have indicated a link between OSAS or IH with increased liver TG accumulation in NAFLD through modulation of β -oxidation of fatty acids and *de novo* lipogenesis in hepatocytes (28-31). In addition, in experimental models of NASH it has been shown that mimicking OSAS with IH has pro-inflammatory effects as indicated by increased levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) (30, 31). Likewise, *in vitro* studies using hepatocytes demonstrated that hypoxia-inducible factor 1 alpha (HIF-1 α) increases hepatocyte apoptosis and generation of pro-inflammatory signals in inflammatory cells (32-34). Moreover, a link between HIF-1 α and hepatic inflammation and fibrosis has also been described in animal studies of IH (35-37). Taken together, the experimental evidence suggests that hypoxia, in a HIF-1 α dependent manner, contributes to the transition from isolated steatosis to more advanced stages of NAFLD and can aggravate lipotoxicity, inflammation and/or fibrosis.

The pathophysiology of NASH is known to involve hepatocellular damage associated with lipotoxicity, triggering local inflammatory responses and contributing to liver fibrosis. However, there is a lack of knowledge regarding the mechanisms of the detrimental effect of hypoxia on fat-laden hepatocytes and the role of intercellular communication between hepatocytes and non-parenchymal cells types such as Kupffer and stellate cells.

Recent studies have indicated that extracellular vesicles (EV) play a key role in intercellular communication in liver pathobiology (38-40). In particular, EV have been implicated in hepatocellular injury, inflammation and hepatic fibrosis in NAFLD, both in humans as well as in experimental models of NASH (41-45).

EV are nanoparticles defined by a lipid bilayer. The cargo of EV can have many effects on target cells, both physiological as well as pathophysiological. Classification of EV depend on their size and site of biogenesis: Exosomes (40-150 nm) are released from multivesicular bodies, microvesicles (MVs) (50-1000 nm) are released from the budding plasma membrane and apoptotic bodies (50-5000 nm) are released from blebbing cells (47). Exosomes and MVs may contain a variety of bioactive molecules, including cytoplasmic proteins, lipids, specific lipid raft-interacting proteins, messenger RNAs, noncoding RNAs and metabolites (39-46). In general, apoptotic bodies are excluded from studies on EV (46). EV have been implicated in many pathophysiological processes and recently, the field has expanded into EV-based diagnostics, prognosis and therapeutics (48).

Several research groups have demonstrated the involvement of EV in intercellular communication in *in vivo* and *in vitro* models of liver diseases (42-44,48,49). Recent studies have shown that hepatocyte-derived EV activate pro-inflammatory signals and pro-fibrotic signals in non-parenchymal cells (48,

49). Additionally, increased levels of circulating EV in *in vivo* models of NAFLD correlate with histologic features of NASH that indicate liver damage (42-44,50). Therefore, it is important to study the content of EV that exert paracrine effects on target cells. Interestingly, recent data suggest that EV released from fat-laden hepatocytes activate the inflammasome via caspase-1 activation in hepatocytes and macrophages leading to an inflammatory response (51).

The NOD-like receptor Pyrin Domain Containing 3 (NLRP3) inflammasome is specific and critical for the activation of caspase-1 and the processing of pro-inflammatory cytokines. The NLRP3 inflammasome has recently been demonstrated to contribute to the transition from NAFLD to NASH (52,53). Studies have also shown that expression of inflammasome components is increased in mouse models of NASH and in humans with NASH (54,55). Moreover, downregulation of NLRP3 or caspase-1 inflammasome components alleviate hepatic steatosis, inflammation and fibrosis (55-58), suggesting that the inflammasome is a potential therapeutic target in NASH.

The research presented in this doctoral thesis investigates whether hypoxia exacerbates lipotoxicity in fat-laden hepatocytes and liver injury in a mice model of non-alcoholic steatohepatitis via the release of extracellular vesicles and cellular crosstalk between hepatocytes and non-parenchymal cells. Additionally, we examined inflammasome activation in conditions of hypoxia, both *in vitro* and *in vivo*. Taken together, the results of this thesis establish a link between extracellular vesicles and hepatocellular damage in hypoxia models that mimics OSA. This link involves EV-mediated intercellular communication between hepatocytes and non-parenchymal cells in non-alcoholic steatohepatitis.

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Chapter 2

EXTRACELLULAR VESICLES IN NAFLD/ALD: FROM PATHOBIOLOGY TO THERAPY

ABSTRACT

In recent years, knowledge on the biology and pathobiology of extracellular vesicles (EV) has exploded. EV are submicron membrane-bound structures secreted from different cell types containing a wide variety of bioactive molecules [i.e., proteins, lipids, and nucleic acids (coding and non-coding RNA, and mitochondrial DNA)]. EV have important functions in cell- to-cell communication and are found in a wide variety of tissues and body fluids. Better delineation of EV structure and advances in the isolation and characterization of their cargo have allowed to explore diagnostic and therapeutic implications of these particles. In the field of liver diseases, EV are emerging as key players in pathogenesis of both alcoholic liver disease (ALD) and nonalcoholic liver disease (NAFLD), the most prevalent liver diseases worldwide and their complications, including development of hepatocellular carcinoma. In these diseases, stressed/damaged hepatocytes release large quantities of EV that contribute to the occurrence of inflammation, fibrogenesis and angiogenesis that are key pathobiological processes in liver disease progression. Moreover, the specific molecular signatures of released EV in biofluids have allowed to consider EV as promising candidates to serve as disease biomarkers. Also, different experimental studies have shown that EV may have potential for therapeutic use as a liver-specific delivery method of different agents taking advantage of their hepatocellular uptake through interactions with specific receptors. In this review, we will focus on the most recent findings concerning the role of EV as new structures mediating autocrine and paracrine intercellular communication in both ALD and NAFLD as well as their potential use as biomarkers of disease severity and progression. Emerging therapeutic applications of EV in these liver diseases are also examined and the potential for successful transition from bench to clinic.

1. - INTRODUCTION

Knowledge of the pathobiology of extracellular vesicles (EV) has expanded significantly in the last decade (1, 2). Indeed, significant advances have been made in delineating the mechanisms of assembly and release of EV as well as their subsequent membrane fusion with target cells (3, 4). Moreover, powerful analytical techniques have made possible the extensive characterization of the cargo of EV that includes a myriad of molecules including growth factors, metabolic enzymes, microRNAs and transcription factors, certain proteins, lipids and metabolites, among others, that modulate intercellular and inter-organ communication (3, 5, 6). Of note, high-throughput datasets of vesicular components are now available in public databases, which strongly supports EV research (7, 8).

Novel insights into the biology of EV show that these particles regulate critical biological functions and may act as contributors to disease pathogenesis and may also serve as disease biomarkers in virtue of the relative simplicity of EV isolation from different biofluids (9). In addition, EV are gaining interest from a therapeutic point of view due to their potential as a unique drug delivery system (10).

In the field of hepatology EV have recently emerged as novel players in the pathogenesis and progression of several conditions (11-13) including the two most common liver diseases world-wide: non-alcoholic liver disease (NAFLD) and alcoholic liver disease (ALD) (14). Specifically, recent studies point to a significant role of EV in modulating injury, amplifying inflammation and promoting liver fibrosis in both NAFLD and ALD (15-17). Since information on this topic is dynamic and rapidly evolving, we aim to provide an up-to-date overview of the current knowledge on the role of EV in the context of both NAFLD and ALD with emphasis on their potential diagnostic and therapeutic impact in these diseases. We exclude from this review data regarding EV in liver cancer since this has been recently reviewed elsewhere (18, 19).

2. - GENERAL CONCEPTS OF EV IN THE LIVER: EV BIOGENESIS, SECRETION AND CARGO

Details on the formation and secretion pathways of EV have been recently reviewed elsewhere (15, 20, 21) and can also be found in other contributions in this special issue of Cells (22). Only basic concepts will be provided here as well as information on aspects that are of particular importance for liver physiology and pathophysiology.

In general, EV are classified according to size and biogenetic pathway, such as exosomes, microvesicles and apoptotic bodies (23). Exosomes are bilayer lipid vesicles with a diameter of 30-150 nm that are derived from endosomal multivesicular bodies (MVBs) (15, 23). Its formation results from the invagination of the plasma membrane (early endosome) and the subsequent fusion of endocytic vesicles mediated by the endosomal sorting complex responsible for transport (ESCRTs) and other components (such as ceramides and tetraspanins) (24). The MVBs can release the intraluminal vesicles known as exosomes by the fusion of MVBs to the plasma membrane, a process mediated in part by Rab GTPases (25). Microvesicles (MVs) have a diameter of 50-1000 nm and originate from the plasma membrane by budding and fission followed by release into the extracellular space (15, 23). MVs contain a subset of cell surface proteins depending on the composition of the parental plasma membrane (26, 27). Apoptotic bodies have a diameter of 100-5000 nm and originate from the budding of cell membranes and may contain nuclear material, which is quickly phagocytosed during programmed cell death (15, 28). Unlike exosomes and MVs, the role of apoptotic bodies is not related to intercellular communication. Therefore, when studying EV under cellular damage conditions, apoptotic bodies are excluded (29).

All EV transport a variety of bioactive molecules, including cytoplasmic proteins, lipids, specific lipid raft-interacting proteins, messenger RNA (mRNA), microRNA (miRNA), ribosomal RNA (rRNA), transfer RNA

(tRNA), noncoding RNAs (ncRNAs), DNA, mitochondrial DNA (mtDNA) and metabolites (24, 30). Lipidomic analysis has shown that EV, independently of their biogenesis, contain a myriad of lipids such as cholesterol, sphingomyelin, ceramide, saturated fatty acids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (4, 23, 29, 30). In addition, proteomic analysis has shown that EV contains different types of proteins, such as heat shock proteins (Hsp70 and Hsp90), tetraspanins (CD9, CD63, CD81, CD82), endosomal sorting complex proteins required for transport (Alix and Tsg101), receptors including epidermal growth factor receptor (EGFR), membrane trafficking proteins (GTPases, Flotillin and Annexins), cytoskeletal proteins (tubulin and actin) and cytosolic proteins (5, 26). Of note, the cargo of EV varies depending not only on their cellular origin but also on the condition under which they are released (i.e. physiological vs. pathological).

In the liver, both parenchymal (hepatocytes) and non-parenchymal cells (i.e. hepatic stellate cells, endothelial, cholangiocytes, Kupfer cells and liver endothelial cells) have been found to release EV in both physiological and pathological states (20). However, information on target cell repertoire, receptors or other specific actions is still limited and incomplete. It has been shown that healthy hepatocytes produce limited amounts of exosomes containing proteins potentially relevant for cell survival, growth and proliferation (31), whereas stressed hepatocytes boost exosome release (32) and enrich their content in specific proteins, lipids and microRNAs that modulate the transcriptional program of neighboring hepatocytes and non-parenchymal cells, thus modulating inflammation and fibrosis which are critical for the progression of liver diseases (Figure 1). Interestingly recent evidence suggests that EV from fat-laden hepatocyte can also signal to other organs such as adipose tissue influencing adipogenesis and tissue remodeling (33).

2.2 EV and liver inflammation

Hepatocellular damage determines the release of a number of signals into the extracellular environment that can contribute to tissue inflammation (34). Some of these signals (collectively termed damage-associated molecular patterns [DAMPs]) are packaged in EV and signal between hepatocytes and non-parenchymal cells such as liver-resident macrophages (Kupffer cells, KC) (35). Indeed, EV may evoke synthesis and release of proinflammatory cytokines such as pro-interleukin (IL)-1b and IL-6 (36) by KCs, thus contributing to local inflammation (37). Also, EV released from hepatocytes can promote the recruitment of additional immune cells (i.e. proinflammatory monocyte-derived macrophages) into the liver maintaining and amplifying inflammation. EV can also signal to endothelial cells and can contribute to vascular inflammation (38). Furthermore, to add complexity, EV can also be secreted from other cells and influence liver inflammation. In this regard, some evidence suggests that platelet-derived EV may have

proinflammatory effects in the liver but this needs further confirmation (39, 40). Finally, it has also been shown that EV are released from monocytic cells and induce polarization towards the anti-inflammatory M2 phenotype of neighboring naive monocytes by delivering cargo miR-27a, thus contributing to resolution of inflammation (41). Collectively, these findings suggest that EV released from injured hepatocytes have an important role in modulating the inflammatory response during liver damage through intercellular communication between different cell types with potential contributions from other cell-derived EV (35). Specific mechanisms involved in NAFLD and ALD are reviewed below.

2.3 EV and liver fibrosis

Persistent liver fibrogenesis and the development of cirrhosis are responsible for the liver-related morbidity and mortality associated with chronic liver diseases (42). Activation or trans-differentiation of HSCs resulting in insoluble collagen deposition and distortion of the normal macro- and micro-anatomical structure of the liver is the major driver of liver fibrogenesis (43). The role of paracrine signals originating from injured epithelial cells (hepatocytes) that can directly or indirectly induce HSC activation has been recognized in recent years (44). In this regard, EV seem to play a role as shown by previous reported studies. Of note, lipid-induced hepatocyte-derived EV seem to regulate HSC activation by shuttling specific microRNAs (i.e. miR-128-3p) that suppress PPAR- γ expression in HSC leading to a marked increase of profibrogenic gene expression (45). Other authors have shown that internalization of endothelial-derived exosomes by HSCs enhances cell migration in a process mediated by sphingosine 1-phosphate (S1P) (46). Additionally, intercellular communication between quiescent and activated HSCs via exosomes can also modulate fibrosis. In this regard, a role for shuttled microRNA 214 (miR-214) in regulating the expression of alpha-smooth muscle actin and collagen in activated HSCs has been demonstrated (47, 48). Moreover, EV derived from non-resident cells such as platelets or granulocytes have been shown to increase angiogenesis and to have procoagulant properties, thus promoting fibrogenesis (49, 50). These findings suggest that EV appear to be key modulators in fibrosis as signals from both parenchymal and non-parenchymal liver cells can either drive or slow down HSC activation. In addition, circulating EV may be used as a biomarker of hepatic fibrosis and have potential implications for the development of novel anti-fibrotic targets (51) as reviewed in the following sections.

3. - EV AS BIOMARKERS IN LIVER DISEASES

Dynamic changes of EV generation in pathological conditions and the accessibility to measure and analyze them in biological samples (i.e. blood, urine, bile and other biofluids) render EV good candidates as disease biomarkers. The relative accessibility for testing and performing repeated measurements over time could facilitate early diagnosis, disease monitoring and development of personalized medicine.

Furthermore, refinement of techniques allowing isolation and in depth characterization of EV cargoes can lead to identification of disease-specific molecular signatures or profiles and provide ample opportunities for EV to be used as suitable, non-invasive biomarkers (52).

EV have been studied as potential biomarkers of liver injury in the setting of ALD, NAFLD, drug-induced liver disease and cholangiopathies (13, 27, 53, 54) as well as diagnostic tools for liver cancer (i.e. hepatocellular carcinoma and cholangiocarcinoma) (19, 54). In this regard, determination of the number of circulating EV and the use of nucleic acid based, lipid-based and protein-based diagnostics have been performed to measure EV enriched in liver-derived DNA, microRNAs, lipids or proteins (13, 27). However, before implementing EV-based biomarkers in the clinic, standardization of sample processing (i.e. collection, transportation, storage and handling) and assay systems is needed as well as large replicative studies to allow EV molecular signatures to be conclusively linked to specific liver diseases. Recent advances related to NAFLD and ALD are reviewed below.

4. - THERAPEUTIC POTENCIAL OF EV

From a therapeutic perspective, EV either unmodified or engineered, can be utilized for therapeutic purposes (55). With regard to liver diseases, efforts have been focused on two major areas: a) the use of EV as delivery vehicles of drugs to the liver (56) and b) the use of EV themselves as therapeutic agents to stimulate liver regeneration, modulate inflammation, reduce liver fibrosis or halt hepatocarcinogenesis (57, 58). The former approach involves the use of different techniques to load EV with a desired cargo (i.e. miRNA, siRNA, chemotherapeutic agents) to act as a “Trojan horse” on target cells. Theoretically, membrane properties of EV allow organ and cell-specific delivery, immune-evasion and targeting of distinct intracellular trafficking pathways. However, a number of challenges related to the manufacturing of EV (i.e. production, coating, loading, etc.) still need to be solved before controlled clinical studies can be carried out (59, 60). With regard to the use of EV as therapeutic agents, most of the available evidence has been generated using mesenchymal stem cell (MSC)-derived EV obtained from human umbilical cord or human embryos that have been tested in numerous preclinical liver disease models (i.e. carbon tetrachloride, thioacetamide, D-galactosamine/TNF- α -induced lethal hepatic failure and bile duct ligation) with promising results. Data for NAFLD and ALD are more limited and are reviewed in the corresponding sections below.

A third, and still nascent, approach related to EV-based therapy is based on the concept that interfering with EV secretion or uptake may attenuate harmful effects on target cells (11, 61). In this regard, several pharmacological agents are being explored that have been shown to inhibit EV trafficking, modify lipid metabolism or decrease EV secretion. However, the complexity of EV biogenesis poses significant challenges to the development of specific agents able to block EV production selectively [see ref. (61) for an in-depth review of this topic].

5. - EV IN NAFLD

A number of studies have demonstrated a role of EV in both pathogenesis and progression of NAFLD (14, 15). Triggering of inflammation and fibrosis development are key for progression from isolated steatosis (also referred as NAFL or non-NASH fatty liver) to nonalcoholic steatohepatitis (NASH), which is hallmarked by the presence of hepatocyte ballooning as reflect of ongoing liver injury and death (36). Data from different diet-induced animal models of NASH have shown that EV concentration increases with disease progression in a time-dependent manner.(17, 62, 63). This seems to be a response to the accumulation of toxic lipids and their downstream mediators in the liver, which increase the capacity of hepatocytes to form and release different types of EV (37, 62-64). *In vitro* treatment of hepatocytes with non-esterified fatty acids evokes the release of EV containing numerous molecules including C-X-C motif ligand 10 (CXCL10), sphingosine-1-phosphate (S1P), mitochondrial DNA (mtDNA), micro-RNAs, ceramides and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (15). These molecules may amplify inflammation through multiple mechanisms such as macrophage activation and monocyte chemotaxis as well as inflammasome activation and modulation of the NF- κ B pathway in target cells (64, 65). As mentioned earlier, EV may be released by different mechanisms including a caspase-3-dependent mechanism (63) or activation of death receptor 5 (DR5) in hepatocytes. Hepatocyte-derived EV are able to induce expression of pro-inflammatory cytokines and promote M1 polarization of hepatic macrophages (37, 66). Of note, CXCL10-bearing EV can also serve as chemotactic stimuli for macrophages as shown recently (64). Moreover, EV released from hepatocytes can contribute to hepatic recruitment of monocyte-derived macrophages by promoting monocyte adhesion via integrin β 1 (ITG β 1)-dependent mechanisms as shown in murine NASH (67). Additional stimuli can also stimulate EV release from hepatocytes. In this regard, it has been observed that hypoxia determines that fat-laden hepatocytes release EV able to signal KC evoking proinflammatory phenotypes in this cells, a phenomena that may explain may underlie the aggravating effect of obstructive sleep apnea syndrome on NAFLD (68). Thus, it seems clear that lipotoxic injury of hepatocytes determines EV release, promoting inflammation through activation and recruitment of macrophages (14) with clear implications for the triggering of inflammation in NAFLD/NASH (36). In addition, hepatocyte-derived EV may promote HSC activation (45, 69) in experimental models of NAFLD/NASH. Interestingly, both mouse and human HSCs release EV that target hepatocytes and HSCs themselves (47, 48, 70). Collectively, these data implicate EV as part of the cellular events triggering hepatic fibrogenesis, a key process in NAFLD progression (71). In addition to signaling to macrophages and HSCs, EV may act on endothelial cells (38, 63) promoting vascular inflammation with potential implications for NAFLD-related atherosclerosis. In this regard, it has been shown that EV carrying miR-1 as cargo mediates proinflammatory effects in endothelial

cells in mice via downregulation of KLF4 and activation of the NF- κ B pathway (38). Finally, other organ-derived EV (i.e. visceral adipose tissue-derived exosomes) can contribute to NAFLD pathogenesis and progression and influence fibrogenic pathways in both hepatocytes and HSCs (72) underscoring the role of other insulin-sensitive organs in NAFLD.

Studies carried out in mouse models of NASH have shown that total circulating EV and particularly hepatocyte-derived EV are elevated early in the disease process while other cell-derived EV (i.e. macrophage- and neutrophil-derived EV) appear in the circulation later, likely reflecting the ongoing inflammatory process (62, 73). Proteomic profiling of circulating EV in experimental NAFLD has been demonstrated to allow differentiation between NAFLD vs. control animals (17). These findings underscore the potential of EV as minimally invasive biomarkers for NAFLD (74), which are urgently for clinical trials and in the clinic. Since circulating EV (mainly exosomes) are also increased in human NAFLD and have been found by some authors to correlate with disease histological features (75), EV analysis in serum involving quantitative and qualitative determinations (including cell surface markers assessment and measurement of different cargoes [i.e. proteins, lipids and microRNAs]) is now a focus of intense research. Several studies have been published in this regard showing that the number of CD14+ and CD16+ EV is inversely associated with the severity of NAFLD-related liver fibrosis, while also increasing the diagnostic capability of the enhanced liver fibrosis score (LFS) in patients with NAFLD (AUC: 0.948 and 0.967 for CD14+ and CD16+ EV, respectively, vs 0.915 for LFS alone) (76). Other efforts include detection of circulating EV containing C16:0 ceramide- and S1P-enriched lipid species that progressively increase in the plasma of obese patients with simple steatosis and in NASH patients with early fibrosis (62). Unfortunately, diagnostic accuracy of these determinations remains incompletely explored in the field of NAFLD/NASH and rigorous validation of this approach is needed (13, 74). Moreover, significant challenges remain regarding isolation, reproducibility and definition of normal controls (13, 77).

Therapeutic efforts involving EV in the field of NAFLD/NASH are nascent. Attempts to halt inflammation and fibrosis in rodent models of NAFLD/NASH using EV as therapeutic agents have been published recently. EV obtained from amnion-derived MSC (AMSC) to treat rats with either NASH or liver fibrosis induced by the hepatic toxicant CCL4. AMSC-EV were given intravenously in one or two doses and amelioration of inflammation and fibrogenesis was observed (78). More recently, human liver stem cells (HLSCs)-derived EV have been used to treat mice with diet-induced steatohepatitis (79). The authors found that EV-HLSC treatment significantly downregulated hepatic pro-fibrotic and pro-inflammatory gene expression and ameliorated the histological abnormalities in mice with NASH. Proteomic analysis of EV-HLSCs showed that their cargo included various anti-

inflammatory proteins such as Interleukin-10, that may contribute to the observed beneficial effects. These results underscore the concept that EV can be exploited for therapy in NAFLD/NASH.

6. - EV AND ALD

Recent studies have focused on the role of EV in ALD (11, 14, 80, 81). Both hepatocyte and monocyte-derived EV have been postulated to regulate macrophage differentiation, thereby promoting inflammation in alcoholic hepatitis (AH) (24, 41, 53, 69, 82). Several molecules have been proposed to be responsible for EV-mediated cell-to-cell signaling, including miRNAs (in particular, miR-122 and miR-155) (41, 81, 83-88). The CD40 ligand was proposed as an EV cargo that could promote macrophage activation in vitro and in vivo in experimental models of AH (81). Also, in a mouse model of ALD involving gastric infusion of ethanol, a microRNA barcode (let7f, miR-29a, and miR-340) that can be detected in blood, which is specific for alcohol-related liver injury (16).

From a clinical point of view, there is currently no biomarker able to assess early stages of ALD. EV have been shown to correlate with the diagnosis and prognosis of alcoholic hepatitis (89). Additionally, EV have been proposed as potential biomarkers to differentiate mild and severe forms of ALD and their cargo, such as sphingolipids, to discriminate between different liver disease etiologies (89). Early identification of these subjects might lead to timely intervention in the disease process. At present, the diagnosis of AH relies on a history of alcohol consumption in a compatible clinical scenario. There are no biomarkers which permit the early diagnosis of AH in “at-risk” populations nor screening tests that can anticipate those at risk for more severe manifestations of AH. This at-risk group is particularly important to identify since there is no effective therapy for severe AH and efforts to avoid it could be implemented. Several clinical prognostic markers have been proposed but the variables used reflect the severity of liver disease. These indices (i.e. the Maddrey discriminant function, model for end-stage liver disease (MELD) and the Lille Score) are based on the non-specific biochemical assessment of liver and renal function and rely heavily on serum total bilirubin, prothrombin time, and creatinine (90). The reliance on bilirubin limits their diagnostic utility, as specificity is confounded by hyperbilirubinemia in co-existent cholestatic liver diseases such as drug-induced liver disease or cholestatic hepatitis such as primary sclerosing cholangitis and primary biliary cholangitis. None of these prognostic scores utilizes a pathophysiologically validated biomarker that reflects the underlying molecular and signaling mechanisms of the disease. Furthermore, the inflammatory response is predictive of mortality but is not taken into account in mathematical models. They can identify subjects at highest mortality risk with an area under the receiver operating curve (AUROC) of under 0.8; ideal survival models should have an AUROC >0.8 (91). The reason these scores are imperfect may be that they are not based on pathophysiologic mechanisms that mediate liver injury. Liver biopsy, the gold standard for diagnosis of AH, remains underutilized due to the concurrent coagulopathy, which greatly

increases the risk of biopsy-related complications (92). Liver biopsy-based hepatic histology score performs as an AUROC of 0.73 in predicting 90-day mortality (91). In this regard the use of EV as biomarker in AH is promising. Thus, it has been recently reported that the total number of EV was significantly increased in patients with AH and that two microRNAs (i.e. miRNA-192 and miRNA-30a) were significantly increased in plasma of subjects with AH (82). Most recently, EV have been used as a surrogate marker of improvement in clinical trials of patients with AH (93). Further studies will be required to validate EV as a biomarker for AH diagnosis and/or prognosis.

7. - CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The field of EV in fatty liver diseases is rapidly evolving as the important functions of these particles in cell-to-cell communication and in the pathogenesis of both ALD and NAFLD, the most prevalent liver diseases worldwide, are unveiled. The release of large quantities of EV by stressed/damaged hepatocytes contributes to inflammation, fibrogenesis and angiogenesis, fueling liver disease progression and provides opportunities for intervention. The identification of specific molecular signatures of released EV is promising in the search for disease-specific biomarkers although more data is needed to validate these markers in larger cohorts and in a rigorous manner. EV may have potential for therapeutic use but this field is still nascent. More research is needed for a successful transition of current EV knowledge from the bench to the clinic.

Figure 1. Extracellular vesicles (EV) can be released by hepatocytes upon lipotoxic or alcohol-induced injury. EV cargoes include a myriad of molecules that can act on target cells evoking inflammatory and fibrogenic events and promoting neoplastic transformation thus contributing to the progression of both alcoholic liver disease (ALD) and nonalcoholic liver disease (NAFLD) to their inflammatory and more aggressive forms nonalcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH).

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Chapter 3

CHEMICAL HYPOXIA INDUCES PRO-INFLAMMATORY SIGNALS IN FAT-LADEN HEPATOCYTES AND CONTRIBUTES TO CELLULAR CROSSTALK WITH KUPFFER CELLS THROUGH EXTRACELLULAR VESICLES

ABSTRACT

Background: Obstructive sleep apnea syndrome (OSAS), which is characterized by occurrence of intermittent hypoxia (IH), is an aggravating factor of non-alcoholic fatty liver disease (NAFLD). We investigated the effects of hypoxia in both in vitro and in vivo models of NAFLD. **Methods:** Primary rat hepatocytes treated with free fatty acids (FFA) were subjected to chemically induced hypoxia (CH) using the hypoxia-inducible factor-1 alpha (HIF-1 α) stabilizer cobalt chloride (CoCl₂). Triglyceride (TG) content, mitochondrial superoxide production, cell death rates, pro-inflammatory cytokines and inflammasome components gene expression and protein levels of cleaved caspase-1 were assessed. Also, Kupffer cells (KC) were treated with conditioned medium (CM) and extracellular vesicles (EV) from hypoxic fat-laden hepatocytes and the choline deficient L-amino acid defined (CDAA)-fed mice model used to assess the effects of IH on experimental NAFLD in vivo. **Results:** CH induced a 2-fold increase in HIF-1 α protein levels. Hepatocytes exposed to FFA and CoCl₂ exhibited increased TG content and higher cell death rates as well as increased mitochondrial superoxide production and mRNA levels of pro-inflammatory cytokines and of inflammasome-components interleukin-1 β , NLRP3 and ASC. Protein levels of cleaved caspase-1 increased in CH-exposed hepatocytes. CM and EV from hypoxic fat-laden hepatic cells evoked a pro-inflammatory phenotype in KC. Livers from CDAA-fed mice exposed to IH exhibited increased mRNA levels of pro-inflammatory and inflammasome genes as well as increased levels of cleaved caspase-1. **Conclusion:** Hypoxia promotes inflammatory signals including inflammasome/caspase-1 activation in fat-laden hepatocytes and contributes to cellular crosstalk with Kupffer cells by release of EV. These mechanisms may underlie the aggravating effect of OSAS on NAFLD.

1. - INTRODUCTION

In recent years, obstructive sleep apnea syndrome (OSAS), a common sleep disorder characterized by recurrent closure of the upper airways during sleep, has been suggested to modulate the severity of different metabolic disorders (1, 2). The hallmark of OSAS is the occurrence of intermittent hypoxia (IH) leading to tissue hypoxia and promoting oxidative stress, inflammation and a myriad of multi-organ pathophysiological effects (3). Among conditions in which OSAS acts as both a potential inducer and as an aggravating factor is non-alcoholic fatty liver disease (NAFLD), the commonest liver disease worldwide (4-6). NAFLD describes a clinicopathological entity defined by the presence of a spectrum of hepatic histological changes ranging in severity from isolated steatosis (also termed non-alcoholic fatty liver [NAFL]) to steatohepatitis (named non-alcoholic steatohepatitis, NASH) through

to advanced fibrosis and cirrhosis (7, 8). Development of steatosis is closely linked to both overweight and obesity as well as to insulin resistance and transition from isolated steatosis to more advanced stages of the disease occurs only in a minority of NAFLD patients. Multiple factors influence NAFLD development and progression including individual's genetic background, environmental factors and the presence of a myriad of comorbidities including OSAS (6, 9).

The main mechanisms of hepatocellular damage in NAFLD include those related to lipotoxicity, mitochondrial dysfunction, formation of reactive oxygen species, endoplasmic reticulum stress and disturbed autophagy ultimately leading to hepatocyte injury and death that triggers hepatic inflammation, hepatic stellate cell activation, and progressive fibrogenesis, thus driving disease progression (8, 10-12). In addition, recent studies have shown that inflammasome activation is also one of the key events in NAFLD progression (13-15). Specifically, the NOD-like receptor Pyrin Domain Containing 3 (NLRP3) inflammasome has been recognized to be involved in the progression of liver damage in experimental *in vitro* (hepatocytes and immune liver cells) and *in vivo* models of NASH (16-18) and also in human studies (19). NLRP3 inflammasome mediates the maturation of inactive pro-caspase-1 into active cleaved caspase-1, which cleaves gasdermin D (GSDMD) that in turn determine the activation of the pro-inflammatory cytokines interleukin [IL]-1 β and IL-18, which amplify the pathological phenomena in NAFLD by promoting inflammation and pyroptotic cell death (20). Of note, the progression of damage in NAFLD also involves the participation of other liver-resident cells such as macrophages or Kupffer cells (KC) as well as the recruitment of inflammatory cells from the periphery (21-23).

With regard to the pathophysiological connections between OSAS and NAFLD, existing data indicate that OSAS may relate to both NAFLD occurrence as well as disease progression. Of note, several studies have shown that IH is able to induce metabolic alterations such as insulin resistance and increased liver triglyceride (TG) accumulation as well as increased oxidative stress and increased inflammation, which are related to steatosis development and hepatocellular damage, respectively (24-26). Hepatic TG accumulation in the setting of OSAS may result from hypoxia-related changes in lipid metabolic pathways such as a decrease in fatty acid β -oxidation and increased *de novo* lipogenesis (5, 27). Also, IH has been shown to promote pro-inflammatory effects in animal models of NAFLD modulating inflammatory cytokine production (i.e. tumor necrosis factor-alpha [TNF- α] and IL-6) (28, 29). Although some studies suggest a relevant role of hypoxia, likely through induction of the hypoxia inducible factor 1 alpha (HIF-1 α), in promoting hepatocellular cell death and the generation of pro-inflammatory signals in several experimental settings (30), assessment of these phenomena in the context of NAFLD has been less explored (5, 31).

In the present study, we aimed to assess the effects of hypoxia on cellular lipid accumulation, hepatocellular death and pro-inflammatory signals including those related to the NLRP3 inflammasome leading to caspase-1 activation in *in vitro* and *in vivo* models of NAFLD. Moreover, we explored whether hypoxia modulates cellular crosstalk between hepatocytes and resident macrophages (i.e. KCs) in the setting of NAFLD and if this crosstalk involves the release of extracellular vesicles (EV) from hepatocytes. Of note, EV have been recently recognized as playing an important role in amplifying the inflammatory response in NASH (32, 33) but few data exist on the potential role of hypoxia in modulating their release. We found that hypoxia induces hepatocellular damage in fat-laden hepatocytes that involves NLRP3 inflammasome-associated caspase-1 activation and increased mitochondrial superoxide production leading to increase cell death rates by multiple mechanisms including apoptosis and pyroptosis. Also, hypoxia contributes to evoking a pro-inflammatory response in Kupffer cells by a mechanism that involves EV release from fat-laden hepatocytes. These observations partially clarify the mechanisms underlying the aggravating effect of OSAS on NAFLD.

2. - MATERIALS AND METHODS

2.1 Animals

Specified pathogen-free male Wistar rats (220–250 g; Charles River Laboratories Inc., Wilmington, MA, USA) and male C57bl6 mice [purchased from Jackson Laboratories (Bar Harbor, ME, USA)] were used in the present study. Animals were housed under standard laboratory conditions with free access to standard laboratory chow diet and water. All experiments were carried out according to the Dutch and Chilean laws on the welfare of laboratory animals and guidelines of the local institutional animal care and use committees of the Pontificia Universidad Católica de Chile and ethics committee of University of Groningen for care and use of laboratory animals. All efforts were made to minimize animals suffering and to reduce the number of animals used.

2.2 Cell isolation techniques, culture conditions, cell surface markers detection and use of liver-derived cell lines

To conduct the experiments described below we used both primary rat hepatocytes and KC. Isolation techniques used are described elsewhere (34, 35) and in the Supplementary material file. Details of culture conditions and KC cell surface markers detection are also provided in that section. The human hepatocellular carcinoma cell line HepG2 (American Type Culture Collection [ATCC] HB-8065, Manassas, VA, USA) was used to carry out experiments involving EV isolation after treatment with FFA. Culture conditions are described in the supplementary material file.

2.3 Treatment with free fatty acids and chemical hypoxia induction

In order to assess the effects of hypoxia in fat-laden liver cells, hepatocytes were incubated with a mixture of free fatty acids (FFA) consisting of oleic acid (500 $\mu\text{mol/L}$) and palmitic acid (250 $\mu\text{mol/L}$) in an aqueous solution of BSA as described (36). Incubations were carried out with or without Cobalt (II) chloride (CoCl_2 ; Sigma Aldrich, Saint Louis, MO, USA) (200 $\mu\text{mol/L}$) for 24 hours to induce chemical hypoxia (37). CoCl_2 is a well-known hypoxia-mimetic agent, that mimics hypoxia/ischemic conditions by stabilization of hypoxia-inducible factor HIF-1 α (38). Control cells were treated with BSA alone. To obtain the corresponding conditioned medium (CM), after treatment, hepatocyte culture medium was replaced by FBS-free medium for an additional 24 hours. CM from different treatments were added to KC for 24 hours in order to explore the possibility that hypoxia modulates hepatocytes-KC cellular crosstalk.

2.4 Oil red O cell staining and TG measurement

Primary rat hepatocytes cultured on glass cover slides were treated with FFA and CoCl_2 for 24 h. Hepatocytes were then washed with phosphate-buffered saline (PBS) twice, and then fixed with 4% formalin for 10 minutes. Cells were washed with 60% isopropanol twice and stained with oil-red-O solution for 10 minutes at room temperature. Then, cells were washed with 60% isopropanol and washed in distilled water for 5 minutes. Then cells were incubated with hematoxylin/eosin solution for 1 minute and washed with distilled water for 5 minutes. Slices were mounted using 1 drop of glycerin-gelatin solution. Stained lipid droplets in cells were examined using a slide scanner, NanozoomerTM (Hamamatsu Photonics K.K., Shizuoka, Japan). Intracellular TG content was determined using TG Quantification Assay kit (ab65336, Abcam, Cambridge, UK) according to the manufacturer's instructions and normalized to protein concentration.

2.5 Apoptosis measurement

Caspase-3 fluorometric assay was used to determine apoptosis induced by FFA and/or CoCl_2 in freshly isolated mouse hepatocytes. After treatment, hepatocytes were scraped and cell lysates were obtained by three cycles of freezing (-196°C) in liquid nitrogen and thawing (37°C) followed by centrifugation for 5 minutes at 13,000g. Caspase-3 enzyme activity was assayed as described previously (39). Arbitrary units of fluorescence (AUF) were quantified in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 430 nm.

2.6 Assessment of cell death associated to disrupted cellular membrane integrity

SYTOXTM Green nucleic acid stain (Invitrogen, S7020, Carlsbad, CA, USA) was used to determine cell death induced by FFA and/or CoCl_2 in hepatocytes (40). Cells were cultured in 12-well plates. After

treatment, diluted SYTOX Green solution (1:40.000/HBSS) was added to the plates for at least 15 minutes at 37°C, 5% CO₂. SYTOXTM Green enters the cell upon loss of membrane integrity and binds to DNA acting as a counterstain that can be analyzed when excited at 488 nm. A Leica fluorescence microscope was used at that wavelength of for detection of necrotic cells, which were quantified using Image J software. Lactate dehydrogenase (LDH) release was assessed as described in the Supplementary materials file. Additionally, we assessed the caspase-cleaved gasdermin-N domain (GSDMD-N) to confirm pyroptotic cell death by Western blot to evaluate the occurrence of pyroptotic cell death (19).

2.7 Mitochondrial superoxide detection

At the end of 24h-long incubations, hepatocytes were washed once with warm HBSS followed by incubation with 200 nmol/L MitoSOXTM Red Molecular Probes (InvitrogenTM, Carlsbad, CA,USA) in William's E medium for 15 min at 37°C protected from light in order to detect mitochondrial superoxide production (41). Then, cells were washed with warm HBSS and mounted onto glass slides using DAPI staining solution (InvitrogenTM, Carlsbad, CA,USA). The fluorescence analyses were immediately recorded using a fluorescent microscope at a wavelength of 510/580 nm (Ex/Em) by a Leica microscope (Leica Microsystems GmbH, Wetzlar, Germany). Quantification of fluorescence in microscopic images of MitoSOX was performed using Image J software.

2.8 Western Blot analyses and antibodies

Protein levels expression were detected by total cell lysate subjected to western blot as previous described (42). The following antibodies were used: Monoclonal mouse anti-HIF1 α 1:1000 (Abcam, USA); monoclonal mouse anti-GSDMD^{C1} 1:500 (Santa Cruz Biotechnology, Dallas, TX, USA); polyclonal rabbit anti-CASP-3 (Cell Signaling Technology, Leiden, The Netherlands); monoclonal mouse anti-CASP1 1:500 (Santa Cruz Biotechnology, Dallas, TX, USA); monoclonal mouse anti-CD81 1:1000 (InvitrogenTM, Carlsbad, CA,USA) and monoclonal rabbit anti-Bcl-2 1:1000 (Abcam, UK) were used in combination with appropriate peroxidase-conjugated secondary antibodies. Monoclonal mouse anti-tubulin or actin were used as loading controls (Sigma, Life Sciences, Merck KGaA, Darmstadt, German). Blots were analyzed in a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Protein band intensities were quantified by ImageLab software (BioRad, Hercules, CA, USA).

2.9 RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated with TRI-reagent (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. Quantification of RNA was measured with the Nanodrop

spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription (RT) was performed using 2.5 µg of total RNA. Quantitative Real Time PCR (qRT-PCR) was carried out in a StepOnePlus™ (96-well) PCR System (Applied Biosystems, Thermofisher, Waltham, MA, USA) using TaqMan method or SYBR Green method. The sequences of the probes and primer sets are described in the Supplementary materials file. mRNA levels were normalized to the housekeeping gene 18S and further normalized to the mean expression level of the control group. Relative gene expression was calculated via the 2^{-ddCT}.

2.10 Enzyme-linked immunosorbent assay (ELISA) of Interleukin-1beta

Levels of pro-inflammatory cytokine of IL-1β in primary rat KC were assessed by the ELISA kit (ab100768, Abcam, Cambridge, UK), according to the manufacturer's instructions.

2.11 EV isolation and characterization

EV were collected from culture media of HepG2 cells as described previously (43) and summarized in the Supplementary materials file. Nanoparticle tracking analysis (NTA) was performed using NanoSight NS300 instrumentation (Marvel, Egham, UK) that uses both light scattering and Brownian motion analysis for nanoparticle characterization. We further characterized EV using Transmission electron microscopy. Sample preparation and details of these analysis are described in the Supplementary materials file.

2.12 Treatment of KC cells with and extracellular vesicles

Rat primary KC were incubated with FBS-free William's E medium and exposed to 15 µg of EV that were isolated from HepG2 cells treated with CoCl₂ + FFA for 24h. After 24h of EV treatment, KC were harvested to continue with analyses by quantitative PCR.

2.13 Effects of intermittent hypoxia in experimental NASH

Animal experiments were approved by the institutional animal care and use committee (Comité de ética y bienestar Animal, Escuela de Medicina, Pontificia Universidad Católica de Chile, CEBA 100623003). Male C57BL/6 mice aged 10 weeks at the beginning of the study and divided into four experimental groups (n = 4–8) receiving either choline-deficient amino acid-defined (CDAA) diet (Catalog # 518753, Dyets Inc. Bethlehem, PA) to induce NASH or the choline-supplemented L-amino acid defined (CSAA, Catalog # 518754, Dyets Inc. Bethlehem, PA) diet as control for 22 weeks as previously described (16, 44). Animals were exposed to IH or normoxia (chambers 41x22x35 cm, COY lab products™, Grass Lake, MI, USA) during the last 12 weeks of the experimental or control feeding period. IH regimen consisted in 30 events/hour of hypoxic exposures for 8 hour/day during the rest

cycle, between 9 am and 5 pm. This cycle was repeated 7 days a week for 12 consecutive weeks. After ending the study, mice were anesthetized (ketamine 60 mg/kg plus xylazine 10 mg/kg intraperitoneally) and then euthanized by exsanguination. Serum and liver tissue samples were collected and processed or stored at -80°C until analyzed. Gene expression and protein analyses were carried out as described above. Positive control of hypoxia induction was evaluated by measurement of hepatic gene expression of HIF-1 α .

2.14 Histological Studies

Liver steatosis was analyzed on paraformaldehyde-fixed liver sections stained with Oil Red-O staining that show lipid deposits in red color on frozen 7 μm liver cryosections. A blinded pathologist (J.T.) assigned a score for steatosis, inflammation and fibrosis as described (44). Scores were given as it follows: Steatosis: grade 0, none present; grade 1, steatosis of $\leq 25\%$ of parenchyma; grade 2, steatosis of 26–50% of parenchyma; grade 3, steatosis of 51–75% of parenchyma; grade 4, steatosis of $\geq 76\%$ of parenchyma and inflammation: grade 0, no inflammatory foci; grade 1, 1–5 inflammatory foci per high power field; grade 2, >5 inflammatory foci/high power field. Fibrosis was estimated qualitatively in Sirius red-stained samples.

2.15 Hepatic triglyceride determination

Hepatic triglyceride content (HTC) was using 40-50 mg of homogenized liver tissue in 1.5 ml of a $\text{CHCl}_3\text{-CH}_3\text{OH}$ mixture (2:1, v/v), followed by a Folch extraction described previously (16, 31).

2.16 Statistical Analyses

Analyses were performed using GraphPad software (version 5.03, GraphPad Software Inc., CA, USA). Statistical analyses were performed using one-way analysis of variance (ANOVA) with a post-hoc Bonferroni correction with multiple-comparison test or by parametric t-tests when needed. All the results are presented as a mean of at least 3 independent experiments $\pm$ SEM. Values were represented as absolute number, normalized data respect to control or percentage for categorical variables. Regarding in vitro assays, independent experiments means different cell plates harvested from at least three animals. Regarding in vivo assays, independent experiments means at least three different animal samples. All the results were analyzed and plotted using GraphPad software (version 5.03, GraphPad Software Inc., CA, USA). Statistics with a value of $p < 0.05$ were considered significant.

3. - RESULTS

3.1 Hypoxia induces HIF-1 α and promotes the increase of lipid droplets in fat-laden hepatocytes: To investigate whether hypoxia exacerbates lipotoxicity in an *in vitro* model of experimental NASH, we

treated fat-laden primary rat hepatocytes with CoCl_2 , a hypoxia mimetic agent that promotes the accumulation of HIF-1 α (45, 46). As expected, chemical hypoxia increased protein levels of HIF-1 α in both control and fat-laden hepatocytes as determined by Western Blot technique (Figure 1). Interestingly, we also observed an increase in the number of intracellular lipid droplets, determined by Oil Red O staining (Figure 2a) and of hepatocyte TG content (Figure 2b) after treatment with a mixture of FFA, which was higher in cells concomitantly treated with CoCl_2 indicating that the hypoxic condition promotes an increase intracellular lipid deposition in this model.

Figure 1. Chemical hypoxia stabilizes HIF-1 α . Primary hepatocytes were incubated for 24 h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. Protein levels of HIF-1 α was determined by Western Blot as described in Material and Methods. α -Tubulin was used as loading control. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$ and ** indicates $P < 0.01$.

Figure 2. Steatosis *in vitro*: Free fatty acids and chemical hypoxia treatment increase the content of triglycerides. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl₂ 200 μmol/L. a) Oil Red O staining (scale bar: 40 μm) and b) Triglycerides (TG) content were measured as described in Materials and Methods. Data were shown as mean ± SEM (n ≥ 3) *** indicates P < 0.005 and **** indicates P < 0.001.

3.2 Hypoxia increases apoptotic and pyroptotic cell death in fat-laden hepatocytes: To evaluate whether the induction of chemical hypoxia exacerbates lipotoxic cell damage and death, we determined protein levels and activity of caspase-3 to assess apoptotic cell death and used SYTOXTM Green nucleic acid stain and LDH leakage to evaluate cell death associated to disrupted cellular membrane integrity. Additionally, we assessed the caspase-cleaved gasdermin-N domain (GSDMD-N) to evaluate pyroptotic cell death (19). While CoCl₂ did not influence cleaved caspase-3 and caspase 3 activity in normal hepatocytes, steatotic hepatocytes undergoing chemical hypoxia displayed a two-fold increase in cleaved caspase-3 (Figure 3a) and six-fold increase in caspase-3 activity (Figure 3b) compared to control cells. Of note, treatment with a mixture of FFA alone led to a two-fold increase (n.s.) in caspase 3 activity suggesting that chemical hypoxia exacerbates lipotoxicity and apoptotic cell death in fat-laden hepatocytes. Also, using SYTOXTM Green nucleic acid stain (Figure 4a) and measurement of cellular LDH leakage (Figure 4b), we found that chemical hypoxia also increased cell death rate associated to losing plasma membrane integrity of fat-laden hepatocytes compared to control cells or cells treated only with FFA. Furthermore, protein levels of GSDMD-N, which is considered a pyroptosis executor (19), increased three-fold in hypoxic fat-laden hepatocytes compared to control cells (Figure 4c).

Figure 3. Chemical hypoxia promotes apoptotic cell death evaluate by a) cleaved Caspase-3 and b) Caspase-3 activity in fat-laden hepatocytes. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. a) Cleaved Caspase-3 protein levels and b) Caspase-3 activity were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM (n $\geq$ 3) * indicates $P < 0.05$; *** indicates $P < 0.005$ and **** indicates $P < 0.001$.

Figure 4. Hypoxia promotes pyroptotic cell death evaluate by a) SYTOX green, b) Lactate dehydrogenase leakage and c) Gasdermin-N in fat-laden hepatocytes. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. a) Sytox green (Representative images and quantification), b) LDH leakage percentage and c) GSDMD-N protein levels determined by Western Blot were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$ and ** indicates $P < 0.01$.

3.3 Hypoxia increases superoxide production by mitochondria in steatosis in vitro: Recent studies on the pathogenesis of NASH have described an important role for mitochondrial oxidative stress (47-50). To evaluate this issue, we examined the levels of mitochondrial superoxide generation in fat-laden hepatocytes treated with CoCl₂. Fat-laden hepatocytes undergoing hypoxia exhibited ten-fold higher levels of mitochondrial superoxide fluorescence determined by MitoSOX™ compared to control cells. CoCl₂ or FFA treatment alone did not determine significant changes in MitoSOX™ fluorescence intensity (Figure 5). These data indicate that chemical hypoxia in fat-laden hepatocytes promotes oxidative stress by an increase of mitochondrial superoxide production.

Figure 5. Hypoxia increases mitochondrial superoxide levels in steatotic hepatocytes. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. Superoxide generation by mitochondria was determined using MitoSOX™ fluorogenic probe as described in Materials and Methods. Data were shown as mean $\pm$ SEM (n $\geq$ 3) ** indicates P < 0.01 and *** indicates P < 0.005.

3.4 Effect of CoCl₂-induced chemical hypoxia on the expression of pro-inflammatory cytokines and inflammasome components in fat-laden hepatocytes: To evaluate whether chemical hypoxia promotes an inflammatory phenotype in fat-laden hepatocytes, we measured mRNA levels of the pro-inflammatory cytokines TNF- α , IL-6 as well as of NLRP3 inflammasome components in cultured cells treated or not with FFA and CoCl₂. As shown in figure 6, steatotic hepatocytes treated with CoCl₂ displayed a marked increase in mRNA levels of IL-1 β (2.5-fold), TNF- α (2-fold), and of IL-6 (9-fold) compared to non-treated cells. Also, mRNA levels of NLRP3 and Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) were also significantly increased in fat-laden hepatocytes undergoing hypoxia compared with cells treated solely with FFA (Figure 6).

Figure 6. Hypoxia increases the expression of pro-inflammatory cytokines in steatotic hepatocytes. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. mRNA levels of IL-1 β , TNF- α and IL-6 were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$ and **** indicates $P < 0.001$.

3.5 CoCl₂-treatment of fat-laden hepatocytes increases protein expression of caspase-1: The inflammasome is a multiprotein complex needed for caspase-1 processing and the subsequent activation of the inflammatory cytokines IL-1 β and IL-18, which has been involved in the pathogenesis of NAFLD/NASH (20). Therefore, we examined the protein expression of caspase-1 in both freshly isolated control hepatocytes and hepatocytes treated with FFA as described above treated or not with CoCl₂ in order to explore the effects of chemical hypoxia on inflammasome activation. While FFA treatment did not influence protein expression of caspase-1, CoCl₂ treatment increased the protein levels of pro-caspase-1 (inactive form) and cleaved caspase-1 (p20 and p10 active forms) in hepatocytes irrespective if they were fat-laden or not (Figure 7) These data suggest that chemical hypoxia is able to activate the inflammasome complex in hepatocytes resulting in caspase-1 activation.

Figure 7. Hypoxia increases cleaved caspase-1 protein levels in steatotic hepatocytes. Primary hepatocytes were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. Protein levels of pro-caspase-1 and cleaved caspase-1 (p10 and p20) were determined by Western Blot as described in Materials and Methods. α -Tubulin was used as loading control. The sum of p20 and p10 caspase-1 was calculated to determine the generation of cleaved Caspase-1. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$ and *** indicates $P < 0.005$.

3.6 Conditioned medium of steatotic hepatocytes subjected to hypoxia increases pro-inflammatory cytokines in Kupffer cells:

To explore whether hypoxic, fat-laden hepatocytes can trigger pro-inflammatory responses in KC, we performed experiments exploring the effects of exposing KCs to CM obtained from fat-laden hepatocytes undergoing chemical hypoxia. We first profiled isolated KC assessing the expression of two typical macrophage markers [CD-68 as macrophage marker and CD-163 as KC marker (51)] by immunofluorescence (Figure 8) confirming the quality of KC isolation. Then, we observed that CM from steatotic hepatocytes subjected to hypoxia increases KC's mRNA levels of pro-inflammatory genes and decreased mRNA levels of the anti-inflammatory cytokine IL-10, without affecting gene expression of the classic KC M2 marker gene arginase-1 (Arg-1) (Figure 9a). Furthermore, protein levels of IL-1 β were significantly higher in KC treated with CM from fat-laden hepatocytes undergoing chemical hypoxia compared with all other groups (Figure 9b). Treatment of cultured rat KC with CM obtained from hepatocytes treated with FFA alone also resulted in higher protein levels of IL-1 β in KC compared to control cells (Figure 9b) but this effect

was less intense than that observed with CM from steatotic hepatocytes undergoing chemical hypoxia, suggesting that hypoxia exacerbates the expression of this pro-inflammatory cytokine. These data support the hypothesis that hypoxic and steatotic hepatocytes release signals that promote a pro-inflammatory phenotype in KC.

Figure 8. Characterization of Kupffer cells by immunofluorescence. Protein expression of specific Kupffer cell green fluorescent marker CD163 (A) and macrophage red fluorescent marker CD68 (B) were determined by immunofluorescence and nuclei were stained blue with DAPI as described in Materials and Methods (Supplementary materials). Scale bar: 10 μ m

b)

Figure 9. Conditioned medium of steatotic hepatocytes subjected to hypoxia increases a) pro-inflammatory genes and b) IL-1 β protein levels in Kupffer cells. Culture medium of primary hepatocytes was replaced 24 hours after treatment by FFA and CoCl₂-free medium for an additional 24 hours (conditioned medium; CM). Kupffer cells were treated for 24 hours with the CM from hepatocytes. a) mRNA levels of IL-1 β , TNF- α , iNOS, IL-6, IL-10 and Arg-1 and b) IL-1 β protein levels by ELISA were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

3.7 Extracellular vesicles increased in CM from fat-laden hepatocellular cell line exposed to CoCl₂ and promoted pro-inflammatory genes expression in KC:

To explore if EV released from fat-laden hepatocytes undergoing hypoxia play a role in promoting a pro-inflammatory effects in KC, EV were collected from culture media obtained from cultured HepG2 cells. We first explored if CM from HepG2 cells exposed to the different treatments evoked proinflammatory signals in KC. Results of these experiments are shown in Supplementary figure 2. As shown in this figure, similar to the effects observed in experiments involving hepatocytes, CM did determine increased expression of proinflammatory genes in KC, a decrease in the expression of IL-10 and had no effect on Arginase-1 expression. We then explored the effects of HepG2-derived EV on KC and compared the observed effects with the effect of treatment with the EV-free fraction. We first characterized EV by transmission electron microscopy as well as by Western blot detection of the presence of the EV marker CD81 and the absence of the mitochondrial protein Bcl-2 in all EV fractions (Figure 10a and Supplementary Figure 1). Of note, the size and concentration of HepG2-derived EV were determined by NTA. Interestingly, we found an increase of EV in culture media from fat-laden cells exposed to CoCl₂ (6.0×10^{11} particles/mL) compared to culture media from control cells (9.5×10^{10} particles/mL) (Figure 10b). As shown in this figure, the average size of EV obtained from culture media of control

cells was 150 nm while those EV obtained from fat-laden HEPG2 cells undergoing CoCl₂ was 130 nm (n.s.). Next, we examined the response of KCs to treatment with HepG2-derived EV. EV from steatotic HepG2 cells treated with CoCl₂ determined a marked increase in mRNA levels in KC of the following proinflammatory genes: IL-1 β (2.0-fold), TNF- α (2.5-fold), iNOS (1.6-fold) and of IL-6 (2.2-fold) in comparison to treatment with EV from non-treated cells, without affecting significantly gene expression of IL-10 and Arg-1 (Figure 10c). However, EV-free CM, as negative control to evaluate the specific effect of EV, promoted an increased on iNOS gene expression in KC (1.4-fold), demonstrating that CM EV-free may contains soluble substances that induce the expression of this specific pro-inflammatory cytokine. Of note, treatment of KC with EV obtained from HepG2 cells treated with FFA or CoCl₂ alone did not evoke significant changes in KC expression of pro-inflammatory genes (Supplementary Figure 3).

Figure 10. EV obtained from culture media of fat-laden HepG2 cells exposed to CoCl₂ increases pro-inflammatory genes in Kupffer Cells. EV obtained from culture media of fat-laden HepG2 cells exposed to CoCl₂ increases pro-inflammatory genes in Kupffer Cells (KC). Presence of EV was confirmed by electron microscopy (a) transmission and quantified using Nano tracking analysis (b). Free fatty acids (FFA) and CoCl₂ treatment of HepG2 cells determined an increase in the number of EV in cultured media with no significant changes in their size. (c) KC were treated with hypoxic and fat-laden HepG2 cells- derived EV (15 μ g) and EV-free culture media as negative control. mRNA levels of interleukin (IL)-1 β , tumor necrosis factor TNF- α , inducible nitric oxide synthase (iNOS), IL-6, IL-10 and Arginase-1 (Arg-1) were measured as described in Materials and Methods. Data is shown as mean $\pm$ SEM (n $\geq$ 3) * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.005; **** indicates P < 0.001.

3.8 Intermittent hypoxia in an experimental model of diet-induced non-alcoholic steatohepatitis increases the inflammatory phenotype: To validate our *in vitro* findings, we investigated the effect of IH for 12 weeks in an animal model, the CDA diet-induced NASH (16). To evaluate steatosis, liver sections were stained with Oil red O staining (Figure 11a) (Abcam, Cambridge, UK). Steatosis, inflammation and fibrosis was graded blindly by an experienced pathologist (J.T.) according to the NAS score. Specifically, the amount of steatosis (percentage of hepatocytes containing fat droplets) was scored as 0 (<5%), 1 (5–33%), 2 (>33–66%) and 3 (>66%). Although pathological scoring suggested a higher degree of steatosis in animals on CDA diet and exposed to hypoxia compared to control animals and compared to animals on CDA diet and normoxic conditions (Figure 11b), we did not observed significant differences in the hepatic TG levels between the CDA-fed mice groups

(Figure 11c). Also, no differences were found in NAS score when comparing CDAA-fed groups. However, mRNA levels of pro-inflammatory genes and NLRP3 inflammasome components significantly increased in animals with hypoxia on CDAA diet compared to all other groups (Figure 12). Finally, we tested the caspase-1 protein levels, which significantly increases in animals with hypoxia on CDAA diet (Figure 13). With these results, we verified that hypoxia promotes an aggravated the inflammatory phenotype in NASH and specifically influences inflammasome activation.

Figure 11. Intermittent hypoxia increased steatosis in mice with CDAA-induced liver injury determined by Oil Red-O staining, without changes in hepatic triglycerides. Mice were placed on choline-supplemented L-amino acid defined (CSAA) diet as control, or defined diet with choline deficiency amino acids (CDAA) for 22 weeks to induce liver injury and intermittent hypoxia (IH) or normoxia was applied for the last 12 weeks of the diet as described in Materials and Methods. a) Liver sections were stained with Oil Red O and analyzed by a pathologist in a blinded fashion to determine b) steatosis score as described in Materials and Methods. c) Hepatic Triglycerides was measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) ** indicates $P < 0.01$; *** indicates $P < 0.005$ and **** indicates $P < 0.001$.

Figure 12. Intermittent hypoxia in mice with CDAA-induced liver injury increases the expression of pro-inflammatory cytokines and inflammasome components. The mice were placed on choline-supplemented L-amino acid defined (CSAA) diet as control, or defined diet with choline deficiency amino acids (CDAA) for 22 weeks to induce liver injury and intermittent hypoxia (IH) or normoxia was applied for the last 12 weeks of the diet as described in Materials and Methods. mRNA levels of IL-1 β , IL-18, NLRP3, caspase-1, IL-6 and IFN- γ were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

Figura 13. Intermittent hypoxia in mice with CDAA-induced liver injury increases cleaved caspase-1. The mice were placed on choline-supplemented L-amino acid defined (CSAA) diet as control, or defined diet with choline deficiency amino acids (CDAA) for 22 weeks to induce liver injury and intermittent hypoxia (IH) or normoxia was applied for the last 12 weeks of the diet as described in Materials and Methods. Protein levels of pro-caspase-1 and cleaved caspase-1 (p10 and p20) were determined by Western Blot as described in Materials and Methods. α -Tubulin was used as a loading control. The sum of p20 and p10 caspase-1 was calculated to determine the generation of cleaved caspase-1. Data were shown as mean $\pm$ SEM ($n \geq 3$) ** indicates $P < 0.01$ and *** indicates $P < 0.005$.

4. - DISCUSSION

NAFLD represents a highly frequent cause of liver disease, which is closely associated with obesity and insulin resistance (7, 52). Clinical observations suggest that patients with OSAS have a greater predisposition to develop NAFLD and NASH, the aggressive form of NAFLD, which is characterized by the presence of necro-inflammatory and fibrotic phenomena in the liver (5). Several studies have shown that OSAS, mainly through the occurrence of IH, can modulate hepatocellular damage by triggering proinflammatory and profibrotic signals (25, 26), but pathways underlying this effects are ill-defined. The present study used cellular models of NAFLD/NASH to explore potential synergistic interactions between hypoxia and FFA exposure. To that end, the hypoxia mimetic agent CoCl₂ was used to treat fat-laden hepatocytes. Our findings suggest that indeed hypoxia determines a further increase in cellular TG content in FFA treated hepatocytes as well as increased mRNA levels of pro-inflammatory cytokines as well as those of the NLRP3 inflammasome components. These phenomena were also associated with increased mitochondrial superoxide levels and increased rates of cell death due to apoptosis and disruption of cell membrane, likely pyroptosis as suggested by the increase in GSDMD levels. Moreover, conditioned medium obtained from hypoxic fat-laden hepatocytes promoted an inflammatory phenotype in KC, which is known to be decisive for the amplification of the pathological phenomenon of NASH (12, 23). Additionally, in order to determine if the observed effects on KC could be mediated by EV release from hepatocytes, we conducted further experiments in the human hepatoma cell line HepG2. We choose this approach for practical reasons since using this cell line the EV isolation efficiency is high. Our results showed that treatment of HepG2 with FFA and CoCl₂ determines an increase in the release of EV to the medium and that these EV also evoke a pro-inflammatory response in rat KC in line with previous observations showing that released EV from fat-laden hepatocytes can act in in macrophages and contribute to pro-inflammatory response in a paracrine fashion (15).

Finally, we found correlates of our *in vitro* NASH model findings in an animal model of NASH induced by feeding a CDAA diet. In the latter experiments, we also confirmed that IH promotes NLRP3

inflammasome activation in the setting of NASH. Collectively taken, these findings indicate that hypoxia may influence NAFLD development and progression by acting at different levels including promotion of further lipid accumulation as well as enhancement of liver inflammation and hepatocellular death. Of note, recent rodent studies indicate that hypoxia may be also linked to liver fibrosis, a key prognostic feature of NASH (8). In fact, Mersarwi et al. demonstrated that HIF-1 α deletion in hepatocytes protects against the development of liver fibrosis in a mouse model of NAFLD (53). This observation adds to our data and support the concept that hypoxia contributes to NAFLD progression influencing critical steps of the disease.

Experimental data on the effects of hypoxia in NAFLD models is scarce and mainly restricted to whole animal studies (53, 54). No data is available on the effects of hypoxia in cellular models of NAFLD/NASH (36). To assess this, we used the hypoxia mimetic compound CoCl₂, which induces chemical hypoxia by stabilizing HIF-1 α and 2 α (38). This model is well accepted and has several advantages over alternatives such as the hypoxia chamber or a CO₂ incubator with regulated oxygen levels, which are less available, most costly and provide a less stable experimental conditions (38). CoCl₂ has been previously used in normal isolated hepatocytes (38, 45) but not in fat-laden cells underlying the novelty of our approach. Of note, previous studies as well as experiments of our laboratory on hepatocyte cell lines have demonstrated the non-toxic effect of CoCl₂ at concentrations not exceeding 400 μ mol/L for 24 hours as previously described (55). On the other hand, FFA treatment using combination of OA and PA effectively induced TG accumulation in the absence of lipoapoptosis (56). In spite of these advantages and, although CoCl₂-induced chemical hypoxia is a suitable model to assess the effects of hypoxia in cellular models, we acknowledge that this model reproduces only some of the effects generated by a decrease in oxygen supply, which limit the generalizability of our results until they are confirmed in other models.

The observed hypoxia-related increases in intracellular lipid droplets and TG content in fat-laden hepatocytes is consistent with previous studies that indicate that hypoxia, through HIF-1 α , promotes an increase in lipid biosynthesis causing TG accumulation (57, 58). We did not observe a marked increase in lipid content in cells treated solely with CoCl₂, which may relate to length of treatment and experimental conditions. We also did not find an increase in the in vivo CDAA-feeding NASH model. We think that this may be related to the fact that this particular model determines severe steatosis and inflammation (59) and that therefore is difficult to observe subtle changes.

With regard to the effects of chemical hypoxia on cell death of FFA treated hepatocytes, qualitative determination of apoptosis, necrosis and pyroptosis using determination of caspase-3 cleavage, caspase-3 activity, SYTOX green staining, LDH release and GSDMD-N were carried out as performed

in previous studies (56, 60). While, treatment with CoCl₂ or FFA alone did not induce a significant increase in apoptosis compared to control conditions, CoCl₂ treatment of fat-laden hepatocytes did increase cleaved caspase-3 and caspase-3 activity reflecting ongoing apoptosis. The same observation was made regarding cell death associated to a disrupted plasma membrane, by SYTOX Green and LDH leakage measure, as an increased number of necrotic death cells was observed in steatotic hepatocytes subjected to hypoxia. Given the fact that hypoxia was associated to increase of GSDMD cleavage and up-regulation of inflammasome components, it is likely that pyroptosis also contributed to cell death of fat-laden hepatocytes undergoing chemical hypoxia. Of note, it has been suggested that pyroptosis may be an inflammatory link related to the progression from bland steatosis to NASH, as NLRP3 activation is not present in animal model of isolated steatosis without inflammation (61). Additionally, we observed a significant increase in mitochondrial superoxide levels in the fat-laden hepatocytes exposed to chemical hypoxia. Interestingly, increased mitochondrial superoxide generation has been correlated to mitochondrial dysfunction, oxidative damage, cellular death and pro-inflammatory effects in NASH (49, 62). Thus, this synergistic effect of hypoxia and FFA in hepatocytes with regard to mitochondrial superoxide generation, likely contributes to the observed increase in cell death when hepatocytes are exposed to both stimuli.

In addition to the observed effects on cell death and oxidative stress, chemical hypoxia also induced the expression of several inflammatory cytokines, including TNF- α , IL-6 and IL-1 β , as well as of the components of the NLRP3 inflammasome (caspase-1, NLRP3 and ASC), in steatotic hepatocytes. The NLRP3 inflammasome activates pro-caspase-1 into active caspase-1 (p10 and p20 cleaved caspase-1) that in turn cleaves pro-IL-1 β into the mature form of IL-1 β promoting amplification of liver inflammation and cell death (12). In recent years, caspase-1 has been studied extensively and has been shown to contribute to liver damage during the development of NASH (63, 64) and several studies have shown protective effects against liver damage when caspase-1 is inhibited (63). In our study, we evaluated the protein levels of caspase-1 (pro-caspase -1 and active cleaved-caspase-1) in hepatocytes. An increase in the total level of pro-caspase-1 was observed in hypoxic hepatocytes and hypoxic hepatocytes treated with FFA compared to the control conditions. Interestingly, cleaved-p-20 and p-10, the active isoforms of caspase-1, were only detected in hypoxic hepatocytes treated with FFA. These results are consistent with the observed up-regulation of inflammasome components and similar to those obtained by other authors in prostate epithelial cells undergoing hypoxia, showing increased NLRP3 inflammasome activation as determined by increased cellular levels of cleaved caspase-1 (65). Taken together, our results suggest that the induction of hypoxia in hepatocytes promotes susceptibility to damage by FFA, resulting in increased caspase-3 mediated

apoptosis, (secondary) necrosis, pyroptosis and an inflammatory phenotype, characterized by increased expression of pro-inflammatory genes and protein levels of caspase-1.

Kupffer cells (KC) are involved in the pathogenesis of various liver diseases, including steatohepatitis (66). However, their role in the context of hepatocellular damage due to hypoxia remains poorly explored. KC are derived from circulating monocytes and, once established in the liver, fulfill multiple functions related to the immune system. KC maintain constant paracrine communication with neighboring hepatocytes, stellate cells and endothelial cells through the signals that they receive from the extracellular environment (66). To verify the paracrine involvement of KC with steatotic hepatocytes in the context of hypoxia, KC were exposed to conditioned medium of hepatocytes subjected to different treatments. We documented that conditioned medium from steatotic and hypoxic hepatocytes induced a pro-inflammatory profile in KC. These novel results correlate well with recent studies showing micro-RNA-mediated cellular crosstalk between hepatocytes treated with ethanol and monocytes/ macrophages (67). As mentioned before, hepatocytes are able to modulate signaling pathways in macrophages, stellate cells and endothelial cells in a paracrine manner by cytokines, micro-RNAs and EV (32). Our experiments involving isolation of EV from HEPG2 cells strongly suggest that EV participate in the cellular communication between steatotic and hypoxic hepatocytes and KC. This observations may have diagnostic and therapeutic implications (33).

In the present study, we also explored whether IH influences hepatic steatosis and the pro-inflammatory phenotype in an *in vivo* model of NASH. To this end, we assessed the effects of hypoxia for 22 weeks in CDAA-fed mice by evaluating liver histology and hepatic mRNA levels of pro-inflammatory cytokines. Our results showed a worsening of histological steatosis in CDAA-fed mice subjected to IH compared to those animals that did not undergo hypoxic conditions. However, we did not observe a corresponding increase in the hepatic TG content. We think that this may be related to the fact that this particular model determines severe steatosis and inflammation (59) and that therefore is difficult to observe subtle changes.

With regard to inflammatory markers, CDAA-fed mice exposed to IH exhibited an increase of all inflammasome components (IL-1 β , IL-18, NLRP3 and caspase-1) and other pro-inflammatory cytokines (IL-6, IFN- γ). Also, active caspase-1 level protein increased in livers from CDAA-fed mice exposed to IH, which correlates with the findings in the *in vitro* model. These observations support the concept that hypoxia may contribute to liver damage in the setting of NAFLD by activating the NLRP3 inflammasome. A recent study showing that IH activates the NLRP3 inflammasome in kidneys, which result in progressive renal injury (68) provides support to this notion. Although not explored in

this study, this might have implications not only for hepatocellular injury but also for liver fibrosis development and hepatocarcinogenesis (69, 70).

In summary, our results in *in vitro* and *in vivo* models of NAFLD/NASH support the notion that hypoxia plays an aggravating role in the setting of excessive lipid load in liver cells by influencing lipid deposition, hepatocellular death and pro-inflammatory signals, involving NLRP3 inflammasome activation. Moreover, our novel findings demonstrate that hypoxia modulates cellular crosstalk between steatotic hepatocytes and inflammatory cells (KCs). Future studies should focus on the characterization of the extracellular environment of hypoxic and steatotic hepatocytes to identify the hypoxia-specific factors and the mechanisms of hepatocellular damage at play in NASH.

5. - SUPPLEMENTARY FIGURES

Figure S1. Characterization of HepG2-EV. EV were characterized in culture medium from control HepG2 cells by detection of EV-positive marker CD81 and EV negative mitochondrial marker Bcl-2 by Western blot as described in Material and Methods.

Figure S2. Conditioned medium of steatotic HepG2 cells subjected to hypoxia increases pro-inflammatory genes in Kupffer cells. Culture medium of HepG2 cells was replaced 24 hours after treatment by FFA and CoCl₂-free medium for an additional 24 hours (conditioned medium; CM). Kupffer cells were treated for 24 hours with the CM from HepG2 cells. mRNA levels of IL-1 β , TNF- α , iNOS, IL-6, IL-10 and Arg-1 were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) *indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$.

Figure S3. EV obtained from culture media of fat-laden HepG2 cells exposed to CoCl₂ increases proinflammatory genes in Kupffer cells. KC cells were exposed to 15 μ g of EV that were isolated from HepG2 cells that had been treated with CoCl₂, FFA and CoCl₂ + FFA for 24h. mRNA levels of IL-1 β , TNF- α , iNOS, IL-6, IL-10 and Arg-1 were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

Figure S4. Intermittent hypoxia in mice increases the gene expression of HIF-1 α . The mice were placed on choline-supplemented L-amino acid defined (CSAA) diet as control, or defined diet with choline deficiency amino acids (CDAA) for 22 weeks to induce liver injury and intermittent hypoxia (IH) or normoxia was applied for the last 12 weeks of the diet as described in Materials and Methods. mRNA levels of HIF-1 α was measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

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Chapter 4

EXTRACELLULAR VESICLES DERIVED FROM FAT LADEN HYPOXIC HEPATOCYTES PROMOTE A PRO-FIBROTIC PHENOTYPE IN STELLATE CELLS

ABSTRACT

Background: Transition from steatosis to non-alcoholic steatohepatitis (NASH) is a key issue in Non-alcoholic fatty liver disease (NAFLD). Observations in patients with obstructive sleep apnea syndrome (OSAS), suggest that hypoxia contributes to progression to NASH and liver fibrosis and the release of extracellular vesicles (EV) by injured hepatocytes has been implicated in NAFLD progression. **Aim:** to evaluate the effects of hypoxia on pro-fibrotic response and the release of EV in NAFLD and to assess cellular crosstalk between hepatocytes and human hepatic stellate cells (LX-2). **Methods:** HepG2 cells were treated with free fatty acids and subjected to chemically-induced hypoxia (CH) using the hypoxia-inducible factor-1 α (HIF-1 α) stabilizer cobalt chloride (CoCl₂). Lipid droplets, oxidative stress, apoptosis and pro-fibrotic-associated genes were assessed. EV were isolated by ultracentrifugation. LX-2 cells were treated with EV from hepatocytes and pro-fibrogenic-associated markers were determined. CDAA-fed mice model was used to assess the effects of intermittent hypoxia (IH) on experimental NASH. **Results:** CH-treatment in fat-laden HepG2 cells increased the steatosis, oxidative stress, apoptosis, pro-fibrotic gene expression and increased the release of EV compared to non-treated HepG2 cells. Treatment of LX2 cells with EV from fat-laden hypoxic hepatocytes increased pro-fibrotic markers compared to EV from non-treated hepatocytes. CDAA-fed animals exposed to IH exhibited increased fibrosis that correlated with an increase of circulating EV. **Conclusion:** Hypoxia promotes hepatocellular damage and pro-fibrotic signaling that correlates with increases of EV release from hepatocytes and from our *in vivo* model of NASH. EV from hypoxic fat-laden hepatocytes evoke pro-fibrotic responses in LX-2 cells.

1. - INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the most common liver disease and is a major global health problem (1). NAFLD is characterized by fat accumulation in the liver which may progress to hepatitis, cirrhosis and liver-related morbidity and mortality (2). Recent evidence suggests that saturated fatty acids (FFAs) contribute to the phenomenon of lipotoxicity and can trigger inflammation in the liver and an abnormal wound-healing response or fibrosis leading to the development of non-alcoholic steatohepatitis (NASH) (3-5).

It is still not clear why some patients with fatty liver develop advanced stages of NASH more rapidly and severely than others. Clinical observations in patients who suffer from Obstructive Sleep Apnea

syndrome (OSAS) have revealed that these patients have a higher risk of developing more severe NAFLD associated with significant liver damage (6,7). The pathophysiology of this condition is characterized by intermittent airway obstruction that alters gas exchange leading to periodic hypoxia (8). Several studies have demonstrated that hypoxia induces metabolic alterations such as insulin resistance, increased oxidative stress, increased liver triglyceride accumulation and increased inflammation, hepatocellular damage and fibrogenesis (9-14).

During hypoxia, the transcription factor hypoxia inducible factor 1 alpha (HIF-1 α) is stabilized and translocated to the nucleus to activate its target genes by means of binding of HIF-1 α to Hypoxia Responsive Elements (HREs) located in the target gene promoters (15). These target genes modify hepatocyte lipid metabolism and also energy metabolism, cell survival, inflammation and fibrosis (16-18). In rodent models of NASH, intermittent hypoxia was shown to have a pro-inflammatory and pro-fibrotic effect as indicated by increased levels of NF- κ B-dependent inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β , and an increased expression of collagen type I in the liver (20,21). Likewise, *in vitro* studies using hepatocytes and stellate cells showed that HIF-1 α can modulate pro-fibrogenic signaling (17,21-23), which could be key for the development and progression of NASH.

The involvement of extracellular vesicles (EV) in the progression of NAFLD has been studied recently in *in vitro* and *in vivo* models (24-27). EV correspond to small particles released after the fusion of multivesicular bodies or directly from the cell membrane with a size of 50 to 150 nm (28). EV play an important role in cellular communication in normal physiological and pathophysiological situations due to their content of proteins, mRNAs and/or lipids (29). There are enough evidence that indicate the involvement of EV in NASH (30,31), but their role in the context of hypoxia and effects on non-parenchymal cells, notably stellate cells, the principal cell type involved in matrix deposition in fibrogenesis, remains to be elucidated.

Therefore, the aim of this study was to test the hypothesis that hypoxia leads to hepatocellular damage that involves release of EV that have a pro-fibrotic effect on stellate cells. We used both *in vitro* and *in vivo* model to test this hypothesis.

2.- MATERIALS AND METHODS

2.1 Animals

Animal experiments were approved by the institutional animal care and use committee (Comité de ética y bienestar Animal, Escuela de Medicina, Pontificia Universidad Católica de Chile, CEBA 100623003). Male C57BL/6 mice aged 10 weeks at the beginning of the study and divided into four experimental groups (n = 4-8) receiving either choline-deficient amino acid-defined (CDAA) diet

(Catalog # 518753, Dyets Inc. Bethlehem, PA) to induce NASH or the choline-supplemented L-amino acid defined (CSAA, Catalog # 518754, Dyets Inc. Bethlehem, PA) diet as control for 22 weeks as previously described (32,33). Animals were exposed to IH or normoxia (chambers 41x22x35 cm, COY lab products™, Grass Lake, MI, USA) during the last 12 weeks of the experimental or control feeding period. IH regimen consisted of 30 events/hour of hypoxic exposures for 8 hour/day during the rest cycle, between 9 am and 5 pm. This cycle was repeated 7 days a week for 12 consecutive weeks. After ending the study, mice were anesthetized (ketamine 60 mg/kg plus xylazine 10 mg/kg intraperitoneally) and then euthanized by exsanguination. Serum and liver tissue samples were collected and processed or stored at -80 °C until analyzed.

2.2 Histological studies

Liver fibrosis was analyzed on paraformaldehyde-fixed liver sections stained with Sirius Red on frozen 7 µm liver cryosections. Staining was quantitated by digital image analysis (ImageJ, NIH, US) as previously described (32).

2.3 Cell culture and treatment with free fatty acids and chemical hypoxia induction

The human hepatocellular carcinoma cell line HepG2 (ATCC, USA) was cultured in Dulbecco's modified Eagle medium (1X) + GlutaMAX™-I (DMEM, 10569010, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO₂. All cells were plated in a cell culture plate at least 24h-36h before treatment. Upon reaching 80% confluence, the cells were incubated with a mixture of free fatty acids (FFA) consisting of oleic acid (500 µmol/L) and palmitic acid (250 µmol/L) in an aqueous solution of bovine serum albumin (BSA) as described (34). Incubations were carried out with or without Cobalt(II) chloride (CoCl₂; Sigma Aldrich) (200 µmol/L) for 24 hours to induce chemical hypoxia (35). CoCl₂ is a well-known hypoxia-mimetic agent by stabilization of hypoxia-inducible factor (HIF)-1α (36). Control cells were treated with BSA alone.

2.4 Western blot analyses

Cell lysates were resolved on Mini-PROTEAN® TGX Stain-Free™ Precast Gels (BioRad, UK, Oxford). Semi dry-blotting was performed using Trans-Blot Turbo Midi Nitrocellulose Membrane with Trans-Blot Turbo System Transfer (BioRad). Ponceau S 0.1% w/v (Sigma) staining was used to confirm protein transfer. Anti-HIF1α (610958, Biosciences), anti-CASP1 (SC-56036, Santa Cruz), anti-CD81 (10630D, Invitrogen), anti-Bcl-2 (ab32370, Abcam), anti-Type I Collagen (1310-01; Southern Biotech) and anti-αSMA (A5228, Sigma) were used in combination with appropriate peroxidase-conjugated secondary antibodies. Tubulin (T9026, Sigma) or actin (A5228, Sigma) were used as loading controls.

The blots were analyzed in a ChemiDoc XRS system (Bio-Rad). Protein band intensities were quantified by ImageLab software (BioRad).

2.5 Nile red staining

Intracellular lipid droplets in the HepG2 cells were detected with Nile Red fluorescent probe (N1142, Thermo Fisher). HepG2 cells were grown in 96-well plates and treated with FFA and/or CoCl₂. After treatment, the cells were washed twice with PBS and stained with Nile Red solution for 10 min in the dark. The cells were then washed twice with PBS and stained with Hoechst dye (33342, Thermo Fisher). The fluorescence intensity of each well was analyzed with excitation/emission wavelength at 488 nm/550 nm using a microplate reader. The fluorescence images were recorded using an inverted fluorescence microscope

2.6 Determination of reactive oxygen species

The intracellular generation of reactive oxygen species (ROS) in HepG2 cells was monitored with DCFH-DA fluorescent probe. HepG2 cells were grown in 96-well plates and treated with FFA and/or CoCl₂. Cells were washed twice with PBS and incubated with the cell permeable reagent 2',7'-dichlorofluorescein diacetate (DCFDA; Abcam, USA) for 45 min. Cells were then washed twice with PBS and the fluorescence intensity of each well was analyzed with excitation/emission wavelength at 495 nm/529 nm using a microplate reader.

2.7 Apoptosis measurement

Caspase-3 fluorometric assay was used to determine apoptosis induced by FFA and/or CoCl₂ in HepG2 cells. After treatment, HepG2 were scraped and cell lysates were obtained by three cycles of freezing (-80°C) and thawing (37°C) followed by centrifugation for 5 minutes at 13,000g. Caspase-3 enzyme activity was assayed as described previously (37). The arbitrary units of fluorescence (AUF) was quantified in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 430 nm.

2.8 Assessment of cell death associated to disrupted cellular membrane integrity

SYTOX® Green nucleic acid stain (Invitrogen, S7020) was used to determine cell death induced by FFA and/or CoCl₂ in HepG2 (38). Cells were cultured in 12-well plates. After treatment, diluted Sytox Green solution (1:40.000/PBS) was added to the cells for at least 15 minutes at 37°C, 5% CO₂. Necrotic cells have ruptured plasma membranes, allowing entrance of non-cell permeable Sytox Green into the cells and binding to nucleic acids. Necrosis was determined using a Leica fluorescence microscope at a wavelength of 488 nm.

2.9 RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

HepG2 cells were harvested on ice and washed twice with ice-cold PBS. Total RNA was isolated with TRI-reagent (Sigma) according to the manufacturer's instructions. Reverse transcription (RT) was performed using 2.5 µg of total RNA, 1X RT buffer (500 mmol/l Tris-HCl [pH 8.3]; 500 mmol/l KCl; 30 mmol/l MgCl₂; 50 mmol/l DTT), 1 mmol/l deoxynucleotides triphosphate (dNTPs, Sigma), 10 ng/µl random nanomers (Sigma), 0.6 U/µl RNaseOUT™ (Invitrogen) and 4 U/µl M-MLV reverse transcriptase (Invitrogen) in a final volume of 50 µl. The cDNA synthesis program was 25°C/10 min, 37°C/60 min and 95°C/5 min. Complementary DNA (cDNA) was diluted 20X in nuclease-free water. Real-Time qPCR was carried out in a StepOnePlus™ (96-well) PCR System (Applied Biosystems, Thermofisher) using TaqMan probes. The sequences of the probes and primer sets are described in Supplementary Material. For qPCR, 2X reaction buffer (dNTPs, HotGoldStar DNA polymerase, 5 mmol/L MgCl₂) (Eurogentec, Belgium, Seraing), 5 µmol/L fluorogenic probe and 50 µmol/L of sense and antisense primers (Invitrogen) were used. mRNA levels were normalized to the housekeeping gene 18S and further normalized to the mean expression level of the control group.

2.10 Extracellular vesicles isolation

For EV collection, HepG2 cells were grown in culture dishes of 100 mm to obtain 70 ml of serum-free conditioned medium after different treatments for an additional 24 hours. EV were isolated from conditioned medium by differential ultracentrifugation (UCF Thermo-Sorvall 80wx+) according to a modified previous protocol (39). A total volume of 70 ml medium per treatment was depleted of cells and cell debris by consecutive, low-speed centrifugations (2,000 × g for 30 min and 12,000 × g for 45 min). The resulting supernatants were carefully collected and centrifuged for 70 min at 120,000 × g at 4 °C. Pellets from this centrifugation step were washed in PBS, pooled and centrifuged again for 60 min at 100,000 × g at 4°C. For EV collection from animals, serum samples were reconstituted in a total volume of 4.4 mL and were centrifuged at 2,000 × g for 30 min and 10,000 × g for 30 min. The resulting supernatants were carefully collected and centrifuged for 70 min at 120,000 × g at 4°C. Pellets from this centrifugation step were washed in PBS, pooled and centrifuged again for 60 min at 100,000 × g at 4°C. The obtained pellets from HepG2 cells or serum were resuspended in lysis buffer or PBS solution depending on subsequent experiments and stored in aliquots at -80 °C. Protein concentration in EV pellet were measured using BCA protein assay kit (Pierce, Rockford, IL).

2.11 Nanoparticle tracking analysis

Concentration and size distribution of isolated EV were assessed by nanoparticle tracking analysis (NTA) using NanoSight NS300 instrumentation (Marvel, Egham, UK). EV samples were diluted with

PBS to a concentration of 10^8 to 10^9 particles/mL in a total volume of 1 mL. Each sample was continuously run through a flow-cell top-plate set up at 18°C using a syringe pump. At least three videos of 20 seconds documenting Brownian motion of nanoparticles were recorded and at least 1000 of completed tracks were analyzed by NanoSight software (NTA v3.2).

2.12 Transmission electron microscopy

Isolated EV were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. The samples were then placed on Formvar/Carbon 300 Mesh (FCF300-CU, EMS) grid and air dried for 10 min. The grids were first contrasted with uranyl-oxalate solution and then contrasted and embedded in a mixture of 4% uranyl acetate. The grids were air dried and visualized with a Philips Tecnai 12 (Biotwin, Eindhoven, Netherlands) electron microscope at 80 kV. The images were captured with the software Item Olympus Soft Imaging Solutions (Windows NT 6.1).

2.13 Treatment of LX-2 cells with extracellular vesicles

To investigate the crosstalk between EV from HepG2 and LX-2 human hepatic stellate cells, a typical cell line to study hepatic fibrogenesis (40), LX-2 cells were treated with isolated EV. LX-2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO₂. All cells were plated in a cell culture plate at least 24-36 h before treatment. Upon reaching 80% confluence, the cells were incubated with FBS-free medium and exposed to 15 µg of EV that were isolated from HepG2 cells that had been treated with CoCl₂, FFA and CoCl₂ + FFA for 24h. After 24h of EV treatment, LX-2 cells were harvested to continue with analyses by quantitative PCR, Western Blot and immunofluorescence.

2.14 Immunofluorescence microscopy

LX-2 cells were grown on glass cover slips placed in 12-well plates. After treatment, culture media were removed and cover slips were carefully washed twice with PBS. Cells were then fixed using a 4% paraformaldehyde solution in PBS for 10 min at room temperature and washed twice with PBS. Permeabilization was performed by incubation of the samples for 10 minutes in 0.1% Triton X-100 (Sigma). The cells were washed twice with PBS and incubated with 2% BSA (Sigma) in PBS/0.1% Tween 20 (Sigma) solution for 30 minutes to block non-specific binding sites. Goat anti-Type I Collagen (1310-01; Southern Biotech) was used at a dilution of 1:200 in 2% BSA/PBS in a humidified chamber for 1 hour at room temperature. Samples were subsequently washed twice with 2% BSA/PBS. Finally, cells were incubated with rabbit anti-goat Alexa Fluor® 568 at a dilution of 1:500 in 2% BSA/PBS for 30 min at room temperature in the dark. Slides were mounted with ProLong antifade

with DAPI (Molecular Probes) and images were evaluated using fluorescence microscopy and analyzed by Leica ALS AF Software (Leica).

2.15 Statistical analyses

Analyses were performed using GraphPad software (version 5.03, GraphPad Software Inc., CA, USA). All results are presented as a mean of at least 3 independent experiments $\pm$ SEM or as absolute number or percentage for categorical variables. The statistical significance of differences between the means of the experimental groups was evaluated using one-way analysis of variance (ANOVA) with a post-hoc Bonferroni multiple-comparison test; $P < 0.05$ was considered as statistically significant.

3. - RESULTS

3.1 Chemical hypoxia increases lipid droplet content in fat laden hepatocytes: To investigate whether hypoxia exacerbates lipid accumulation in an *in vitro* model of experimental NASH, we treated HepG2 cells with CoCl_2 , a hypoxia mimetic agent that promotes the accumulation of HIF-1 α . HepG2 cells treated with CoCl_2 showed a significant increase of HIF-1 α , independent of treatment with FFA (Figure 1). To induce steatosis, HepG2 cells were treated with a mixture of oleic acid and palmitic acid (FFA) as described previously (41). Hepatocytes showed increased formation of lipid droplets (Figure 2a). Interestingly, a significant increase in the content of lipid droplets was observed in the steatotic hepatocytes treated with the chemical inducer of hypoxia (Figure 2b), indicating that hypoxia increases steatosis in this model.

Figure 1. Chemical hypoxia stabilizes HIF-1 α . HepG2 cells were incubated for 24h with oleic acid and palmitic acid 2:1 (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. Protein levels of HIF-1 α were determined by

Western Blot as described in Material and Methods. α -Tubulin was used as loading control. Data were shown as mean $\pm$ SEM ($n \geq 3$) **** indicates $P < 0.001$.

Figure 2. Hypoxia aggravates FFA-induced steatosis *in vitro*. HepG2 cells were incubated for 24h with oleic acid and palmitic acid 2:1 (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. Lipid droplets content was measured by a) Fluorescence intensity and b) Fluorescence images of Nile Red as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; **** indicates $P < 0.001$.

3.2 Hypoxia increases oxidative stress and apoptotic cell death in fat laden hepatocytes: To evaluate whether the induction of hypoxia exacerbates damage by lipotoxicity, we determined the generation of reactive oxygen species (ROS) and the extent of cell death. The increase in ROS was evaluated using the fluorogenic dye DCF. The results indicated a significant increase in ROS in each of the treatments compared to the control (Figure 3). Furthermore, hypoxia exacerbates the increase in ROS in steatotic HepG2 cells compared to the hypoxic and FFA conditions separately.

Apoptotic cell death was evaluated by measuring the activity of caspase-3. The results demonstrate that hypoxia in steatotic HepG2 cells induces apoptotic lipotoxicity compared to control cells. (Figure 4a). Hypoxia or steatosis alone did not induce apoptotic cell death. Lipotoxicity was also evaluated by Sytox Green assay to detect cell death associated to disrupted cellular membrane integrity. The different treatments in HepG2 cells did not induce any necrotic cell death (Figure 4b).

Figure 3. Hypoxia increases the production of reactive oxygen species in steatotic HepG2 cells. HepG2 cells were incubated for 24h with oleic acid and palmitic acid 2:1 (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. Reactive oxygen species (ROS) were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

Figure 4. Hypoxia and steatosis in vitro promote apoptotic cell death but do not promote necrotic cell death. HepG2 cells were incubated for 24h with oleic acid and palmitic acid 2:1 (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. Caspase-3 activity and necrosis were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$

3.3 Hypoxia induces the expression of pro-fibrotic cytokines: To evaluate whether hypoxia promotes a profibrotic phenotype in fat laden HepG2 cells, we measured the mRNA levels of different profibrotic cytokines. We demonstrate that chemical hypoxia in fat laden hepatocytes significantly

increases mRNA levels of profibrotic cytokines. Expression of some genes was also increased in hepatocytes treated only with CoCl_2 (Figure 5).

Figure 5. Hypoxia increases the expression of pro-fibrotic cytokines in steatotic HepG2 cells. HepG2 cells were incubated for 24h with oleic acid and palmitic acid 2:1 (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. mRNA levels of TGF- β 1, CTGF, collagen-I, α -SMA and TIMP-1 were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$

3.4 Increased number of EV in hepatocyte conditioned medium in hypoxic and steatotic conditions:

To better characterize the involvement of hepatocytes in the promotion of the pro-fibrotic phenotype and possible crosstalk with stellate cells, we assessed EV from conditioned medium of HepG2 cells (HepG2-EV) following different treatments. After isolation of EV from conditioned medium, we characterized EV according to previous guidelines (42) by their typical structure visualized by TEM (Figure 6a) and detection of EV positive marker CD81 and EV negative marker Bcl-2 (mitochondrial marker) by Western blotting (Figure 6b). Also, the size (around 100-150 nm) and concentration of HepG2-EV (Figure 6c) were determined by NTA. Notably, the fat laden-hepatocytes in the presence of CoCl_2 showed a significant increase in the concentration of HepG2-EV compared to all conditions (Figure 7). These results suggest that steatotic and hypoxic conditions in HepG2 cells can modulate the release of EV.

Figure 6. Characterization of HepG2-EV. EV were characterized in conditioned medium from control HepG2 cells by a) transmission electron microscopy; b) detection of EV-positive marker CD81 and EV negative mitochondrial marker Bcl-2 by Western blot c) size distribution by NTA as described in Material and Methods.

Figure 7. EV release is increased in hepatocyte conditioned medium in hypoxic and steatotic conditions *in vitro*. EV quantification in conditioned medium from HepG2 after different treatments performed by NTA as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$.

3.5 HepG2-EV from steatotic and hypoxic conditions promote a pro-fibrotic phenotype in stellate cells: To evaluate whether HepG2-EV have a direct effect on stellate cells we used the human stellate cell line LX-2. LX-2 cells were stimulated with 15 $\mu\text{g/ml}$ of HepG2-EV from each treatment and mRNA and protein levels of some pro-fibrotic cytokines were evaluated. As shown in Figure 8, HepG2-EV from steatotic and hypoxic condition significantly increase the gene expression of TGF- β -1, CTGF, collagen-I and α -SMA in LX-2 cells. The results also suggest an additive effect of FFA and CoCl_2 to promote a pro-fibrotic phenotype in LX-2 cells. Interestingly, a similar result was observed in a confirmatory experiment assessing protein levels of collagen-I (Figure 9a-9c) and α -SMA (Figure 9b) in LX-2 cells treated with HepG2-EV from steatotic and hypoxic condition.

Figure 8. HepG2-derived EV increase the expression of profibrotic cytokines in LX-2 cells. LX-2 cells were exposed to 15 μg of EV that were isolated from HepG2 cells that had been treated with CoCl_2 , FFA and CoCl_2 + FFA for 24h. mRNA levels of TGF- β 1, CTGF, Collagen-I and α -SMA were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) ** indicates $P < 0.01$; **** indicates $P < 0.001$.

Figure 9. EV derived from steatotic and hypoxic HepG2 cells increases the expression of profibrotic proteins in stellate cells. LX-2 cells were exposed to 15 μg of EV that were isolated from HepG2 cells that had been treated with CoCl₂, FFA and CoCl₂ + FFA for 24h. Protein levels of a) Collagen-I and b) α-SMA were determined by Western blot as described in Materials and Methods. β-Actin was used as loading control. c) Intracellular Collagen-I expression was determined by immunofluorescence as described in Materials and Methods. Data were shown as mean ± SEM (n ≥ 3) * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.005.

3.6 Hypoxia promotes a pro-fibrotic phenotype and correlates with increased release of EV in an *in vivo* model of NASH: To validate the previous results in an *in vivo* model, CDAA diet feeding for 22-weeks was used to induce NASH and an intermittent hypoxia regimen was applied for the last 12 weeks. As expected, CDAA diet-fed mice showed significant liver fibrosis as shown by conventional Sirius Red staining (Figure 10a). Interestingly, collagen deposition area of IH-treated mice with NASH was even further increased compared with CDAA diet-group, indicating a pro-fibrotic action of hypoxia. In addition, as shown in Figure 10b, IH significantly increased hepatic mRNA levels of several markers of fibrogenesis induced by the CDAA diet including TGF- β 1, CTGF, collagen-I, α -SMA, TIMP-1 and MCP-1. Finally, EV were isolated from mice serum to determine the concentration of EV in each experimental group. As shown in Figure 10c, CDAA diet-fed mice exhibited a significant increase of EV when exposed to IH compared to control or CDAA diet-fed mice under normoxic conditions. These results suggest a strong correlation between the induction of hypoxia with the increase of the EV and the promotion of a more pro-fibrotic phenotype in an *in vivo* model of NASH.

a)

b)

c)

Figure 10. Intermittent hypoxia promotes a pro-fibrotic phenotype in mice with NASH and correlates with increased levels of EV *in vivo*. The mice were placed on choline-supplemented L-amino acid defined (CSAA) diet as control, or defined diet with choline deficiency amino acids (CDAA) for 22 weeks to induce liver injury. Intermittent hypoxia (IH) or normoxia was applied for the last 12 weeks of the diet as described in Materials and Methods. a) Liver sections stained with Sirius Red from mice fed control diet, intermittent hypoxia and/or choline-deficient amino acid-defined (CDAA) diet. In addition, fibrosis area was quantified using digital image analysis of the red-stained area in Sirius red-stained samples (ImageJ, NIH, US); mRNA levels of pro-fibrotic cytokines were determined by RT-qPCR as described in Materials and Methods; c) quantification of EV from serum samples was determined by NTA as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

4. - DISCUSSION

In recent years, the mechanisms underlying the progression of NAFLD/NASH have been thoroughly studied. The early stages of NAFLD are characterized by fat accumulation in the liver resulting in steatosis that can progress to hepatocellular damage and inflammation in a condition termed NASH (2,5). Moreover, some patients develop liver fibrosis associating with increased mortality due to risk of HCC development (43). Interestingly, clinical studies have indicated that obstructive sleep apnea (OSA) is a new predictive factor of hepatic fibrosis in patients with NAFLD (44-46). Recent research has provided evidence regarding the role of hypoxia, a hallmark of OSA, in the development of liver injury and hepatic fibrosis in animal models of NAFLD (17,19,47). However, there are still major gaps in our understanding of the progression of NASH to fibrosis, e.g. the cellular mechanism underlying the crosstalk between hepatocytes and stellate cells, the principal non-parenchymal cell responsible for liver scar formation (48). In this study, we demonstrate that hypoxia, induced by a HIF-1 α chemical stabilizer in fat-laden hepatocytes, promotes hepatocellular damage, increases pro-fibrogenic gene expression and increases the release of extracellular vesicles (EV). Furthermore, EV from hypoxic and steatotic hepatocytes promote mRNA and protein expression of important fibrosis markers in stellate cells.

In the present study, our *in vitro* model of steatosis was key to the analysis of hypoxia induced by the hypoxia mimetic CoCl₂, a HIF-1 stabilizer. We used the human hepatocyte cell line HepG2, in which the effectiveness of the hypoxia mimetic was evaluated through the quantification of HIF-1 α levels, which increased due to the intracellular stabilization, as described in other *in vitro* studies (49,50). We showed that treatment with palmitic acid (PA) and oleic acid (OA) (1: 2) increased the content of lipid droplets in hepatocytes in the absence of lipotoxicity. Previous studies have shown that OA promotes steatosis in hepatocytes, both in primary hepatocyte culture and in hepatoma cell lines, while PA induces a cytotoxic response (51,52). Our results are in line with a previous report using a combination of both fatty acids, using a higher OA concentration, that promoted an anti-apoptotic effect and triglyceride accumulation (51). Interestingly, the hypoxia mimetic CoCl₂ increased the content of lipid droplets compared to HepG2 cells without hypoxia, as determined via quantification of Nile Red fluorescence. These results are supported by an *in vivo* study indicating that hypoxia, via HIF-1 α , promotes an increase in lipid biosynthesis in steatotic conditions (53). Hepatocyte cell death by apoptosis was measured using caspase-3 and as expected, treatment with FFA alone did not induce cell death. However, HepG2 cells exposed to the combination of CoCl₂ and FFA presented an increase of caspase-3 activity that correlated with an increase in oxidative stress compared to the control group. Unexpectedly, HepG2 cells treated with FFAs also increased ROS compared to CoCl₂ and control groups. A recent study demonstrated that OA prevents ROS production in HepG2 cells

treated with PA (54). Likewise, another study showed that FFA treatment of HepG2 cells induced TNF- α generation, which is an important mediator in hepatic steatohepatitis and liver injury (55). Additional studies to further delineate the mechanism, such as measurement of mitochondrial ROS production, remain to be performed. We also analyzed hepatocyte cell death associated to disrupted cellular membrane integrity (necrosis) using SytoxGreen®. None of the treatments we applied induced a significant increase in necrosis. The divergent results of apoptosis and necrosis can be explained by the fact that different stimuli may induced different modes of cell death in different cell types (56). Taken together, our findings indicate that hypoxia promotes lipotoxicity as determined by the increase in hepatocellular apoptosis and oxidative stress.

To evaluate the pro-inflammatory effects of hypoxia in fat-laden hepatocytes, we measured gene expression of pro-fibrotic cytokines. As expected and in accordance with our previous results, the condition of hypoxia in steatotic hepatocytes increased the expression of TGF- β 1, CTGF, collagen-I, α -SMA and TIMP-1. Moreover, we checked the amount of EV released in the culture medium and we observed an increase after treatment of HepG2 cells with CoCl₂ and FFA compared to all other groups. EV are small structures surrounded by membrane and released from the cells to the extracellular environment. They have a pathophysiological role in various diseases, including NASH and other chronic liver diseases (30,31,57). We performed a characterization of the isolated EV from HepG2 cells using positive and negative markers by Western blotting and determinate EV size by NTA. Finally, isolated EV from control hepatocytes were directly visualized using TEM.

Previous studies have demonstrated that during hepatocellular damage, hepatocytes release EV that modulate pro-inflammatory and pro-fibrotic signaling in non-parenchymal liver cells (25, 58-60). To confirm whether EV from our model of hypoxic and steatotic hepatocytes promote a pro-fibrotic phenotype in non-parenchymal liver cells, we used the human stellate cell line LX-2. EV derived from CoCl₂-treated fat-laden hepatocytes increased gene expression of pro-fibrotic cytokines such as TGF- β 1, CTGF, collagen-I and α -SMA in LX-2 cells compared to all other groups. In addition, protein expression of collagen-I and α -SMA was analyzed in LX-2 cells treated with EV from HepG2 cells. Both pro-fibrotic proteins increased when LX-2 cells were treated with EV derived from CoCl₂-treated steatotic hepatocytes. This interesting result suggests that the EV-cargo in our model is an important topic for future analysis. Recent studies that support our findings show that palmitic acid increased EV from hepatocytes and change their miRNA cargo inducing pro-fibrogenic signals to HSCs (60). Another study, providing similar results, indicated that EV from lipotoxic hepatocytes activate HSCs via miRNA-128-3p (25).

The exact relation(s) between intermittent hypoxia, as hallmark of OSA, and NAFLD/NASH progression, specifically in development of liver fibrosis, remain incompletely understood. We found that intermittent hypoxia promotes a pro-fibrotic phenotype in our CDAA diet-fed animal model and increases circulating levels of EV. Previous studies have demonstrated that IH mimicking OSA alters EV generation and release as well as EV-cargo and function, promoting pathophysiology in different *in vivo* models (61-63). This important finding correlates with our *in vitro* results and provide evidence for a novel pro-fibrotic mechanism, involving EV. Next challenge will be identified if the most of circulating EV are derived from liver or from other specific cell source and identify gene and proteins cargo, including membrane surface composition.

In conclusion hypoxia promoted hepatocellular damage and increased pro-fibrotic genes that correlates with increases of EV derived from hepatocytes and from our *in vivo* model of NASH. Moreover, EV from hypoxic fat-laden hepatocytes promoted a pro-fibrotic responses in LX-2 cells, showing a novel cell to cell communication in this model. Finally, mice model of NASH exposed to intermittent hypoxia presented a pro-fibrotic phenotype that correlate with increased of circulating of EV. Future work should focus on analysis to profile the genomic, transcriptomic, proteomic and lipidomic cargo into EV-derived from cells and biological fluids from rodents models under hypoxia condition that could be promotes fibrosis profile in the setting of NAFLD and OSAS.

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Chapter 5

HYPOXIA INCREASED CASPASE-1 IN EXTRACELLULAR VESICLES DERIVED FROM EXPERIMENTAL NON-ALCOHOLIC STEATOHEPATITIS MODELS AND PROMOTES INFLAMMASOME ACTIVATION IN KUPFFER CELLS

ABSTRACT

Background: Intercellular crosstalk between hepatocytes and non-parenchymal cells is an important factor in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and the more aggressive form non-alcoholic steatohepatitis (NASH). Extracellular Vesicles (EV) plays an important role in intercellular crosstalk in the context of liver diseases, including NAFLD. Clinical observations have indicated that Obstructive sleep apnea (OSA) is an aggravating factor in NASH. Hypoxia is the hallmark of OSAS, however, whether hypoxia modulates the crosstalk between hepatocytes and non-parenchymal Kupffer cells (KCs) has not been investigated deeply yet. **Aim:** to investigate whether hypoxia modulates hepatocellular damage and intercellular crosstalk in the context of NAFLD and whether EV and inflammasome/caspase-1 are involved in this modulation. **Methods:** Hepatoma cell line HepG2 were treated with free fatty acids (FFAs) and chemical hypoxia (CH) was induced by cobalt (II) chloride (CoCl_2). Pro-inflammatory and inflammasome-related gene expressions were assessed in hypoxic fat-laden HepG2 and in liver tissue. Intermittent Hypoxia (IH) was applied to mice fed a CDAA diet (NASH model). EV were isolated from conditioned medium (CM) or serum by ultracentrifugation and characterized by nanoparticle tracking and electron microscopy. Cleaved caspase-1 and caspase-1 activity was measured by Western blot and enzymatic assay respectively. **Results:** CH in fat-laden HepG2 increased expression of pro-inflammatory and inflammasome-related genes compared to non-treated hepatocytes. These results correlated with the *in vivo* results. The silencing of HIF-1 α using siRNA in HepG2 prevented the increased expression of inflammasome-related genes in KCs. Treatment of KCs with EV from fat-laden hypoxic HepG2 increased pro-inflammatory and inflammasome-related gene expression in KCs compared to KC treated with EV from non-treated HepG2. Cleaved caspase-1 content and activity of caspase-1 was increased in EV from hypoxic fat-laden HepG2 and from serum of CDAA-fed animals exposed to IH. **Conclusion:** Our findings indicate that CH promotes inflammatory phenotype in fat-laden HepG2 and the inflammasome-related genes expression in KCs via HIF-1 α . CH induces the release of caspase-1-EV from fat-laden HepG2 promoting the inflammatory phenotype in KCs. These findings suggest EV and their content (caspase-1) as a potential novel biomarker in NAFLD and OSAS.

INTRODUCTION

Significant advances have been made in understanding the importance of intercellular communication between hepatocytes and non-parenchymal cells in the pathogenesis of liver diseases (1,2). In particular in the pathophysiology of non-alcoholic steatohepatitis (NASH), the inflammatory and progressive form of non-alcoholic fatty liver disease (NAFLD), Kupffer cells (KCs), the liver-resident macrophages, are considered to play a key role in the inflammatory response and disease progression (2,3). Indeed, it has been demonstrated that KC activation promotes steatosis, inflammation and fibrosis in NASH (4-7). An early event in the pathogenesis of NAFLD is the increased accumulation of lipids in the liver, in particular in hepatocytes. This lipid accumulation promotes cytotoxic effects known as lipotoxicity, resulting in hepatocellular damage and triggering of inflammatory signaling (8-9). Emerging data suggest that lipotoxic hepatocytes release extracellular vesicles (EV) to neighboring target cells in an autocrine/paracrine manner and in this way promote liver damage (10-11). EV are 50-150 nanometer-sized vesicles released after the fusion of multivesicular bodies or directly from the cell membrane (12). Their cargo is diverse and may contain proteins, various RNA species and/or lipids (13). Several studies have indicated the importance of EV in the crosstalk between injured hepatocytes and KCs (14-17). Moreover, increased levels of EV have been reported in serum of mice and humans with NASH and these levels correlate with histological liver inflammation and macrophage activation (18-21).

Recent data have also indicated that injured hepatocytes release inflammasome components into the extracellular environment, providing a mechanism to spread inflammasome signaling to adjacent cells (22,23). The activation of the inflammasome promotes the maturation and release of pro-inflammatory interleukins via activation of caspase-1. The active caspase-1 is composed of a tetramer containing two 20 kDa fragments and two 10 kDa fragments (24). The activation of caspase-1 initiates a novel form of programmed necrotic cell death named pyroptosis. Pyroptotic cells release proinflammatory cytokines like IL-1 β and IL-18 and the intracellular enzyme lactate dehydrogenase (LDH) via gasdermin-dependent pores in the plasma membrane (25,26). Recent studies have indicated that caspase-1 activation plays an important role in inflammation and fibrosis in NASH (27,28). A recent study reported the presence of caspase-1 in EV released from monocytes that induced endothelial cell death in a model of lung injury (29). However, the presence and role of caspase-1 in EV in the context of NASH has not been studied yet.

Emerging evidence from clinical studies indicates that intermittent hypoxia (IH), the principal pathophysiological factor in obstructive sleep apnea (OSA), aggravates NAFLD (30). Indeed, there is substantial evidence from rodent models and human subjects that IH associated with OSA increases

hepatic triglyceride (TG) levels and exacerbates inflammation and fibrosis in NASH (31-35). Although the precise molecular mechanisms underlying the aggravating effect of IH on NASH have not been elucidated yet, the activation of the transcription factor hypoxia inducible factor 1 alpha (HIF-1 α) appears to be an important factor. Hepatic HIF-1 α activation increases steatosis and promotes pro-inflammatory and pro-fibrotic signaling pathways (35-38).

Taken together, there is strong evidence that hypoxia via HIF-1 α and Inflammasome/Caspase-1 activation is an aggravating factor in NASH. However, it is still not fully understood whether caspase-1 participates in hypoxia-induced liver injury.

In the present study we have investigated the role of hypoxia and EV in *in vivo* and *in vitro* models of NASH. We demonstrate that hypoxia promotes inflammatory phenotype in fat-laden HepG2 and the inflammasome-related genes expression in KCs via HIF-1 α . Moreover, CH induces the release of caspase-1-EV from fat-laden HepG2 and promotes cellular crosstalk between hypoxic fat laden-hepatocytes and KC.

2. - MATERIALS AND METHODS

2.1 Animals

Specified pathogen-free male Wistar rats (220–250 g; Charles River Laboratories Inc., Wilmington, MA, USA) and male C57bl6 mice [purchased from Jackson Laboratories (Bar Harbor, ME)] were used. Animals were housed under standard laboratory conditions with free access to standard laboratory chow diet and water. All experiments were carried out according to the Dutch and Chilean laws on the welfare of laboratory animals and guidelines of the local institutional animal care and use committees of the Pontificia Universidad Católica de Chile and ethics committee of the University of Groningen for care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Kupffer cell isolation

Kupffer cells were isolated from the non-parenchymal cell fraction obtained during the hepatocyte isolation. The supernatant was subjected to different low-speed centrifugations at 4°C and resuspending the pellets in Hank's Balanced Salt Solution (HBSS) (Gibco, California, USA) supplemented with 0.3% bovine serum albumin (BSA). KC were purified using a 20% of density gradient medium OptiPrep™ (Sigma-Aldrich). After different low-speed centrifugations, the final pellet was dissolved in HBSS supplemented with 10% FBS to allow cells to attach. After the attachment period, KC were cultured in William's E medium supplemented with 50 μ g/ml gentamycin, 10% FBS and 1% of p/s/f for 18 hours until the start of the experiments.

2.3 HepG2 hepatoma cell line

The human hepatocellular carcinoma cell line HepG2 (ATCC, USA) was cultured in Dulbecco's modified Eagle medium (1X) + GlutaMAX™-I (DMEM, 10569010, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO₂. All cells were plated in a cell culture plate at least 24 h -36 h before treatment upon reaching 80% confluence.

2.4 Treatment with free fatty acids and chemical hypoxia

In order to assess the effects of hypoxia on fat-laden liver cells, HepG2 cells were incubated with a mixture of free fatty acids (FFA) consisting of oleic acid (500 µmol/L) and palmitic acid (250 µmol/L) in an aqueous solution of BSA as described (41). Incubations were carried out with or without Cobalt (II) chloride (CoCl₂; Sigma Aldrich) (200 µmol/L) for 24 hours to induce chemical hypoxia (40). CoCl₂ is a well-known hypoxia-mimetic agent, that mimics hypoxia/ischemic conditions by stabilization of hypoxia-inducible factor HIF-1α (42). Control cells were treated with BSA alone.

2.5 Effects of intermittent hypoxia in experimental NASH

Animal experiments were approved by the institutional animal care and use committee (Comité de ética y bienestar Animal, Escuela de Medicina, Pontificia Universidad Católica de Chile, CEBA 100623003). Male C57BL/6 mice aged 10 weeks at the beginning of the study were divided into four experimental groups (n = 4–8) receiving either choline-deficient amino acid-defined (CDAA) diet (Catalog # 518753, Dyets Inc. Bethlehem, PA) to induce NASH or the choline-supplemented L-amino acid defined (CSAA, Catalog # 518754, Dyets Inc. Bethlehem, PA) diet as control for 22 weeks as previously described (43, 44). Animals were exposed to IH or normoxia (chambers 41x22x35 cm, COY lab products™, Grass Lake, MI, USA) during the last 12 weeks of the experimental or control feeding period. IH regimen consisted of 30 events/hour of hypoxic exposures for 8 hour/day during the rest cycle, between 9 am and 5 pm. This cycle was repeated 7 days a week for 12 consecutive weeks. At the end of the study, mice were anesthetized (ketamine 60 mg/kg plus xylazine 10 mg/kg intraperitoneally) and then euthanized by exsanguination. Serum and liver tissue samples were collected and processed or stored at -80 °C until analyzed. Gene expression and protein analyses were carried out as described above.

2.6 Western Blot analyses

Protein lysates were collected by scraping cells in lysis buffer (HEPES 25 mmol/L, KAc 150 mmol/L, EDTA pH 8.0 2mmol/L, NP-40 0.1%, NaF 10 mmol/L, PMSF 50 mmol/L, aprotinin 1 µg/µL, pepstatin 1 µg/µL, leupeptin 1 µg/µL, DTT 1 mmol/L). Total amount of protein in lysates was measured by Bio-Rad protein assay (Bio-Rad; Hercules, CA, USA). For Western blot, 30-50 µg protein was resolved on

Mini-PROTEAN® TGX Stain-Free™ Precast Gels (BioRad, UK, Oxford). Semi dry-blotting was performed using Trans-Blot Turbo Midi Nitrocellulose Membrane with Trans-Blot Turbo System Transfer (BioRad). Ponceau S 0.1% w/v (Sigma) staining was used to confirm protein transfer. anti-CASP-1 (SC-56036, Santa Cruz), anti-CD81 (10630D, Invitrogen), anti-CD63 (sc-5275, Santa Cruz) and anti-ASGPR1 (Novus, USA) were used in combination with appropriate peroxidase-conjugated secondary antibodies. Tubulin (T9026, Sigma) or actin (A5228, Sigma) were used as loading controls. The blots were analyzed in a ChemiDoc XRS system (Bio-Rad). Protein band intensities were quantified by ImageLab software (BioRad).

2.7 RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

After treatment, HepG2 cells and rat hepatocytes were harvested on ice and washed twice with ice-cold PBS. Total RNA was isolated with TRI-reagent (Sigma) according to the manufacturer's instructions. Reverse transcription (RT) was performed using 2.5 µg of total RNA, 1X RT buffer (500 mmol/l Tris-HCl [pH 8.3]; 500 mmol/l KCl; 30 mmol/l MgCl₂; 50 mmol/l DTT), 1 mmol/l deoxynucleotides triphosphate (dNTPs, Sigma), 10 ng/µl random nanomers (Sigma), 0.6 U/µl RNaseOUT™ (Invitrogen) and 4 U/µl M-MLV reverse transcriptase (Invitrogen) in a final volume of 50 µl. The cDNA synthesis program was 25°C/10 min, 37°C/60 min and 95°C/5 min. Complementary DNA (cDNA) was diluted 20X in nuclease-free water. Real-Time qPCR was carried out in a StepOnePlus™ (96-well) PCR System (Applied Biosystems, Thermofisher) using TaqMan probes. The sequences of the probes and primer sets are described in Supplementary Material. For qPCR, 2X reaction buffer (dNTPs, HotGoldStar DNA polymerase, 5 mmol/l MgCl₂) (Eurogentec, Belgium, Seraing), 5 µmol/l fluorogenic probe and 50 µmol/l of sense and antisense primers (Invitrogen) were used. mRNA levels were normalized to the housekeeping gene 18S and further normalized to the mean expression level of the control group. Relative gene expression was calculated using the ΔC_t method. The sequences of the probes and primer sets are described in the Supplementary Material.

2.8 Transfection of HepG2 cell line with HIF-1 α small interfering RNA or control small interfering RNA

HepG2 cells were grown to approximately 60% confluency in 6-well plates before transfection and treatments. Small interfering RNA (esiRNA) was employed to silence HIF-1 α in HepG2 cells (EHU151981, Sigma-Aldrich) using standard Lipofectamine (Lipo3000, Invitrogen) in OPTI-MEM™ I medium at a final concentration of 100 nmol/L according to the manufacturer's protocol. Cells were transfected with EGFP siRNA as control (EHUEGFP, Sigma-Aldrich). Experiments were performed after 24 hours of transfection for 24 hours. The efficiency of transfection was determined by

quantitative RT-PCR and Western blot for HIF-1 α (Supplementary Material). To obtain the corresponding conditioned medium (CM), hepatocyte culture medium was replaced by FBS-free medium after treatment for an additional 24 hours. CM from different treatments were added to KC for 24 hours.

2.9 Extracellular vesicles isolation

For EV collection, HepG2 cells were grown in culture dishes of 100 mm to obtain 70 ml of serum-free conditioned media after 24 hours of treatment. EV were isolated from conditioned medium by differential ultracentrifugation (UCF Thermo-Sorvall 80wx+) according to a modified protocol described previously (46). A total volume of 70 ml medium per treatment was depleted of cells and cell debris by consecutive, low-speed centrifugations (2,000 $\times$ g for 30 min and 12,000 $\times$ g for 45 min). The resulting supernatants were carefully collected and centrifuged for 70 min at 120,000 $\times$ g at 4 °C. Pellets from this centrifugation step were washed in PBS, pooled and centrifuged again for 60 min at 100,000 $\times$ g at 4 °C. For EV collection from serum, samples were reconstituted in a total volume of 4.4 mL and centrifuged at 2,000 $\times$ g for 30 min and 10,000 $\times$ g for 30 min. The resulting supernatants were carefully collected and centrifuged for 70 min at 120,000 $\times$ g at 4 °C. Pellets from this centrifugation step were washed in PBS, pooled and centrifuged again for 60 min at 100,000 $\times$ g at 4 °C. The obtained pellets from HepG2 cells or serum were resuspended in lysis buffer or PBS depending on subsequent experiments and stored in aliquots at -80 °C. Protein from HepG2-derived EV or serum-derived EV were obtained using the Total Exosome Protein Isolation kit (Invitrogen). Protein concentration in EV pellets were measured using BCA protein assay kit (Pierce, Rockford, IL).

2.10 Treatment of Kupffer cells with extracellular vesicles

Rat primary Kupffer cells (KCs) were incubated with FBS-free William's E medium and exposed to 15 μ g of EV isolated from HepG2 cells treated with CoCl₂, FFA or CoCl₂ + FFA for 24h. After 24h of treatment, KCs were harvested.

2.11 Caspase-1 activity

Cell lysate, HepG2-derived EV or serum-derived EV were assayed for their ability to cleave a fluorescent CASP-1 substrate, following the manufacturer's instructions (Abcam, USA).

2.12 Statistical analyses

Analyses were performed using GraphPad software (version 5.03, GraphPad Software Inc., CA, USA). All results are presented as a mean of at least 3 independent experiments $\pm$ SEM or as absolute number or percentage for categorical variables. The statistical significance of differences between

the means of the experimental groups was evaluated using one-way analysis of variance (ANOVA) with a post-hoc Bonferroni multiple-comparison test; $P < 0.05$ was considered as statistically significant.

3. - RESULTS

3.1 Effect of chemical hypoxia on pro-inflammatory, inflammasome-related gene expression and caspase-1 levels in an *in vitro* model of NASH: To investigate whether the HIF-1 α stabilizer CoCl₂ increases the expression of inflammatory genes and inflammasome components in fat-laden liver cells. HepG2 cells were treated with CoCl₂ and FFAs to mimic hypoxia and steatosis *in vitro* as described in Materials and Methods. Figure 1 shows that hypoxia increased expression of pro-inflammatory genes like TNF- α and IFN- γ in fat-laden HepG2 cells. Furthermore, CoCl₂ increased expression of inflammasome components like IL-1 β , IL-18, NLRP3 and Caspase-1, in fat-laden HepG2 cells. Finally, inflammasome activation was determined by measuring cleaved caspase-1 levels in HepG2 cells by Western blot as well as caspase-1 activity. The increased protein level of cleaved caspase-1 (Fig. 2a) and increased caspase-1 activity (Fig.2b) confirmed that the HIF-1 α stabilizer CoCl₂ could promote inflammasome activation in fat-laden HepG2 cells.

Figure 1. CH increases the expression of pro-inflammatory genes and inflammasome components in an *in vitro* model of NASH. HepG2 cells were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl₂ 200 μ mol/L. mRNA levels of TNF- α IFN- γ , IL-1 β , IL-18, NLRP3 and Caspase-1 were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$.

Figure 2. CH increases inflammasome caspase-1 level in an *in vitro* model of NASH a) protein levels of pro-caspase-1 and cleaved caspase-1. b) activity of caspase-1. HepG2 cells were incubated for 24h with oleic acid and palmitic acid (2:1 ratio) (FFA) in the presence or absence of CoCl_2 200 $\mu\text{mol/L}$. Protein levels of pro-caspase-1 and cleaved caspase-1 (p20) were determined by Western blot as described in Materials and Methods. α -Tubulin was used as loading control. Total p20 caspase-1 was calculated to determine the generation of cleaved caspase-1. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; **** indicates $P < 0.001$.

3.2 Silencing of HIF-1 α in hypoxic and fat-laden HepG2 prevents inflammasome activation in KCs:

To explore whether hypoxic and fat-laden HepG2 cells can increase expression of inflammasome-related genes in hepatic resident macrophages, we isolated primary rat Kupffer cells (KC) and investigated whether inflammasome activation in KCs depends on HIF-1 α in fat-laden hepatocytes. Conditioned medium from steatotic HepG2 cells treated with CoCl_2 increased mRNA levels of NLRP3, Apoptosis-Associated Speck-Like Protein Containing CARD (ASC), Caspase-1 and IL-1 β in KC. The increase of these genes was abolished by HIF-1 α knockdown in steatotic HepG2 cells treated with CoCl_2 (Figure 3). Taken together, our results suggest a cellular crosstalk between hepatocytes and macrophages in hypoxia, which is dependent on HIF-1 α activation in hepatocytes, that promotes an inflammatory phenotype in NASH through inflammasome activation.

Figure 3. Silencing of HIF-1 α prevents inflammasome activation in KCs. Culture medium of hypoxic fat-laden HepG2 cells transfected with negative control siRNA (sieGFP) or HIF-1 α siRNA (siHIF-1 α) was replaced after 24 hours of treatment by fresh medium without FFA and CoCl₂. Cells were cultured for another 24 hours and the conditioned medium (CM) was collected. Kupffer cells were treated for 24 hours with CM from HepG2 cells. mRNA levels of NLRP3, Caspase-1, ASC and IL-1 β were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM (n $\geq$ 3) * indicates P < 0.05; ** indicates P < 0.01.

3.3 Extracellular vesicles from steatotic and hypoxic HepG2 cells contain promotes inflammasome activation and inflammatory phenotype in Kupffer cells: Our previous studies have demonstrated that conditioned medium from hypoxic fat-laden HepG2 cells increase the expression of pro-inflammatory cytokines in Kupffer cells (Chapter 3). According with that, we next investigated whether EV from hypoxic and steatotic hepatocytes promote the pro-inflammatory phenotype in KC. EV were isolated from conditioned medium of HepG2 cells (HepG2-EV) following different treatments. KCs were treated with 15 μ g/ml of HepG2-EV from each treatment and gene expression of inflammasome components was evaluated. As shown in Figure 4, HepG2-EV from steatotic hypoxic condition significantly increased gene expression of the inflammasome components NLRP3, ASC, Caspase-1 and IL-1 β in KC (Figure 4). HepG2-EV from steatotic and hypoxic conditions also significantly increased gene expression of IL-6, iNOS and TNF- α (Figure 5). These results suggest a synergistic effect of steatosis and hypoxia on the pro-inflammatory effect of HepG2-derived EV on KCs.

Figure 4. EV from hypoxic, fat-laden HepG2 cells increase the expression of inflammasome components and cytokines in Kupffer cells. Kupffer cells were exposed to 15 μ g of EV isolated from HepG2 cells that had been

treated with CoCl_2 , FFA and $\text{CoCl}_2 + \text{FFA}$ for 24h. mRNA levels of NLRP3, ASC, Caspase-1 and IL-1 β were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

Figure 5. EV from hypoxic, fat-laden HepG2 cells increase the expression of pro-inflammatory cytokines in Kupffer cells. Kupffer cells were exposed to 15 μg of EV isolated from HepG2 cells treated with CoCl_2 , FFA and $\text{CoCl}_2 + \text{FFA}$ for 24h. mRNA levels of IL-6, iNOS and TNF- α were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

3.4 Extracellular vesicles from steatotic and hypoxic HepG2 cells contain increased levels of active caspase-1:

We next investigated whether EV obtained from hypoxic and fat-laden HepG2 cells are enriched in (active) caspase-1. Protein levels of pro-caspase-1 and cleaved caspase-1 (p10 and p20) and the activity of caspase-1 were determined in purified EV. EV isolated from hypoxic fat-laden HepG2 cells contain significantly increased levels of active caspase-1 compared to all other groups (Figure 6).

a)

b)

Figure 6. EV from steatotic and hypoxic HepG2 cells have increased levels of cleaved caspase-1 and caspase-1 activity. a) Western Blot analyses of pro-caspase-1 and cleaved caspase-1 (sum of p10 and p20) and b) the activity of caspase-1 from purified EV were measured as described in Material and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) * indicates $P < 0.05$; *** indicates $P < 0.005$; **** indicates $P < 0.001$.

3.5 Intermittent hypoxia in an experimental model of diet-induced non-alcoholic steatohepatitis increases caspase-1 in circulating EV levels: In order to validate our in vitro findings, we tested whether intermittent hypoxia for 12 weeks in an animal model of CDAA diet-induced NASH increases caspase-1 activation in circulating EV. First, we characterized EV with positive markers, CD63 and CD81 (Figure 7) and then we analyzed the caspase-1 contain EV. We demonstrate that IH in mice with NASH significantly increased caspase-1 levels and activity in circulating EV isolated from mice subjected to IH and NASH compared to EV isolated from all other groups (Figure 8). These finding are correlating with our previous studies which Intermittent hypoxia in mice with CDAA-induced liver injury increases pro-inflammatory signaling, caspase-1 protein levels and caspase-1 activity in the liver (Chapter 3) and increases circulating EV (Chapter 4). Taken together, these results confirm that hypoxia aggravates inflammation in an in vivo model of NASH in a possible mechanism that involves circulating caspase-1-EV.

Figure 7. Extracellular vesicles in a model of diet-induced NASH and intermittent hypoxia. a) Circulating EV were characterized by detection of EV-positive marker CD63 and hepatic positive marker asialoglycoprotein receptor (ASGPR1) by Western blot as described in Material and Methods.

b)

Figure 8. Extracellular vesicles from CDAA diet-fed mice subjected to Intermittent Hypoxia are enriched in caspase-1. a) Western Blot analysis of pro-caspase-1 and cleaved caspase-1 (p10 and p20) and b) the activity of caspase-1 from purified circulating EV were measured as described in Materials and Methods. Data were shown as mean $\pm$ SEM ($n \geq 3$) ** indicates $P < 0.01$; **** indicates $P < 0.001$.

4.- DISCUSSION

The principal finding of this study is that extracellular vesicles from hepatocytes play an important role in intercellular communication in the context of NAFLD. Specifically, we have shown that EV from fat-laden and hypoxic hepatocytes induce a pro-inflammatory phenotype in Kupffer cells, which contributes to the initiation and progression of inflammation in NASH. The induction of this pro-inflammatory phenotype is at least in part mediated by active caspase-1, and possibly other components of the inflammasome, contained within EV.

In recent years, different studies have provided evidence that hepatocyte injury induces a pro-inflammatory response in macrophages via the release of hepatocyte-derived EV, thus contributing to the development and progression of NAFLD (10, 47). The progression from simple steatosis to NASH is not completely understood. The onset and progression of NAFLD from simple steatosis to inflammation (NASH) is most likely the result of multiple factors acting in parallel or sequentially (48). The recent clinical observation that obstructive sleep apnea syndrome (OSAS) is a predictive factor for the progression and aggravation of NAFLD, promoted us to consider hypoxia through HIF-1 α , the hallmark of OSAS, as an important trigger for hepatocellular damage, exacerbating lipotoxicity and inflammation and increasing the release of EV. Previously, we demonstrated that chemical hypoxia, mimicked by the HIF-1 α stabilizer CoCl₂, further increases hepatocellular TG content, pyroptosis-induced cells death and mitochondrial ROS generation in FFA-treated primary hepatocytes and HepG2 cells (10, 11). Now, we complement these finding, adding also that chemical hypoxia increases mRNA levels of inflammasome components in HepG2 cells. These results are in line with our previous studies that hypoxia exacerbates apoptotic cell death determined by caspase-3 and promotes disruption of cellular membranes in fat-laden hepatocytes (10). Interestingly, we

determined cellular crosstalk mechanism-dependent of HIF-1 α in hypoxic fat-laden HepG2 cells on expression of inflammasome-related genes in KCs. This paracrine effect is correlated with our previous studies between hepatocytes with KCs and stellate cells (10, 11). Moreover, we demonstrated an increase in caspase-1 levels contain in EV from conditioned medium obtained from hypoxic fat-laden hepatocytes. These EV promoted an inflammatory phenotype in KCs as evidenced by increased mRNA expression of inflammasome components in EV-treated Kupffer cells. Via this mechanism, Caspase-1-EV may contribute to the inflammation associated with NASH (12-49,50). The inflammasome is an intracellular pro-inflammatory structure that promotes inflammatory signaling and metabolic disruption (51). The activation of inflammasomes involves activation of caspase-1 which in turn cleaves pro-IL-1 β into its active secreted form, thus amplifying the inflammatory response (52). Interestingly, we observed increased caspase-1 activity in EV from fat-laden, hypoxic hepatocytes. In the current study we also demonstrate increased caspase-1 activity in circulating EV in mice with NASH subjected to intermittent hypoxia (IH). Previous studies have demonstrated that circulating EV levels and inflammasome activation correlate strongly with pathophysiological changes observed during NASH development (4,27,28,53). In our previous studies, we also confirmed that IH promotes NLRP3 inflammasome activation and pro-inflammatory and pro-fibrotic signaling in the context of NASH (10,11). In line with these previous results, we have now demonstrated increased levels of circulating EV in mice with NASH and subjected to IH and we also demonstrate that these EV contain a higher activity of caspase-1 compared to circulating EV from control mice. Our results are supported by studies reporting that EV released by hepatocytes exposed to lipotoxic stress may actively contribute to pro-inflammatory responses by activating macrophages in a NLRP3 inflammasome-dependent, paracrine pathway (54-56). In particular, a recent study demonstrated the presence of the inflammasome components NLRP3, pro-caspase-1, pro-IL-1 β and ASC in macrophage-derived EV (57). Taken together, our data extend these observations by demonstrating that EV from hypoxic fat-laden hepatocytes, and in *in vivo* model of hypoxia in experimental NASH, contain active caspase-1 that could induces and extense pro-inflammatory signaling.

In conclusion, our data clearly indicate the importance of crosstalk between hepatocytes and Kupffer cells under steatotic and hypoxic conditions. Moreover, our results suggest that this crosstalk is mediated via activated caspase-1 contained in EV and that this is essential for inflammasome activation in macrophages. Our data provide new insights in intercellular communication and the role of inflammasomes in hepatocyte-derived EV in the context of NAFLD.

Our findings could have implications in the development of EV-based biomarkers, delivery vehicles and therapeutics for the treatment of liver diseases.

5.- SUPPLEMENTARY FIGURES

Figure S1. Silencing of HIF-1α in HepG2 cells . Culture medium of hypoxic HepG2-transfected with negative control siRNA (sieGFP) or HIF-1α siRNA (siHIF-1α). a) Protein levels and b) mRNA levels of HIF-1α decreased compare with treatment of CoCl₂ or CoCl₂ + sieGFP. Values represent mean ± standard deviation from three independent experiments. Data were shown as mean ± SEM (n ≥ 3) * indicates P < 0.05.

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Chapter 6

1. - GENERAL DISCUSSION AND CONCLUSION

NAFLD refers to a spectrum of histological abnormalities in the liver, ranging from isolated steatosis to non-alcoholic steatohepatitis (NASH), the aggressive form of NAFLD, which is characterized by the presence of necro-inflammatory and fibrotic phenomena in the liver (1,2). Understanding the transition from simple steatosis to NASH is a key issue in the NAFLD field. Currently, several studies have shown that OSAS, mainly via the occurrence of intermittent hypoxia (IH), predisposes to the development of NASH (3). Recent studies have also demonstrated that hypoxia can aggravate hepatocellular damage by triggering pro-inflammatory and pro-fibrotic signals (4,5). However, the pathways underlying these effects are still largely unexplored. The present doctoral thesis proposes an important role for extracellular vesicles in the hypoxia-induced exacerbation of hepatocellular damage. We used *in vitro* models of NAFLD/NASH to explore whether hypoxia induces hepatocellular damage, hepatic inflammation and fibrosis via mechanisms that involve cellular crosstalk mediated by extracellular vesicles.

In the Chapter 2, we reviewed the most recent findings concerning the role of extracellular vesicles (EV) in mediating autocrine and paracrine intercellular communication in both ALD and NAFLD as well as their potential use as biomarkers of disease severity and progression (15-17). Considering our results on the crosstalk between hepatocytes and non-parenchymal cells, we propose a role of EV in intercellular communication and the aggravation of damage in our NAFLD models

The principal findings in Chapter 3 suggest that hypoxia promotes lipids droplet accumulation and cell death via apoptosis, necrosis and pyroptosis in fat-laden primary rat hepatocytes. These phenomena are associated with increased oxidative stress and inflammatory signals including inflammasome/caspase-1 activation and contribute to cellular crosstalk between hepatocytes and Kupffer cells through EV, thus aggravating the pathological phenomena of NASH (6,7). Our *in vitro* findings correlated with our findings in an animal model of NASH induced by feeding a CDAA diet. Our results are also in line with previous studies that indicate that hypoxia, via HIF-1 α , promotes lipid accumulation in hepatocytes and contributes to liver inflammation and fibrosis in rodent models of NAFLD (8-10). Our studies are the first to investigate the effects of hypoxia in an *in vitro* model of NAFLD/NASH that involves EV (11). Interestingly, we found a synergistic effect of hypoxia and fat accumulation in hepatocytes on the increased expression of inflammasome components, suggesting that pyroptosis is the major form of cell death observed in these conditions. Moreover, we investigated the effect of hypoxia in an *in vivo* mice model of NASH (CDAA diet). In line with our *in vitro* results, intermittent hypoxia increases steatosis, pro-inflammatory gene expression and hepatic

inflammasome/caspase-1 activation in mice with NASH. It has been shown that caspase-1 activation contributes to liver damage in NASH (12,13).

In Chapter 4 we demonstrated that hypoxia exacerbates hepatocellular damage in HepG2 cells. Furthermore, hypoxia promotes pro-fibrotic signaling that correlates with increased release of EV from hepatocytes in our experimental models of NASH. Moreover, EV from hypoxic fat-laden hepatocytes evoke pro-fibrotic responses in LX-2 cells. These results are in agreement with previous studies demonstrating that EV from lipotoxic hepatocytes induce pro-fibrogenic signals in stellate cells (18,19). Furthermore, we demonstrated increased levels of EV in serum from CDAA-fed animals exposed to hypoxia and these increased levels correlate with histologically determined fibrosis and pro-fibrotic gene expression in the liver. In Chapter 5 we investigated the paracrine effect of EV from fat-laden hypoxic hepatocytes on Kupffer cells through Caspase-1 like cargo into EV. The expression of pro-inflammatory and inflammasome-related genes was increased in Kupffer cells treated with EV from hypoxic fat-laden hepatocytes compared to EV from non-treated hepatocytes, similar finding that we describe in Chapter 3. These results correlate well with previous results indicating crosstalk between injured hepatocytes and Kupffer cells that involves the participation of EV (20-23). We also performed experiments to silence HIF-1 α in hypoxic fat-laden HepG2 cells, to establish the role of HIF-1 α in the pro-inflammatory crosstalk between hypoxic fat-laden hepatocytes (HepG2 cells) and Kupffer cells. We demonstrated that conditioned medium from hypoxic fat-laden HepG2 cells induced an increase in the expression of inflammasome-related genes in Kupffer cells via a mechanism that requires activation of HIF-1 α . These novel results are in line with a recent study that proposes a paracrine crosstalk between Kupffer cells and hepatocytes that promotes pro-inflammatory signaling and leads to inflammatory injury (14). Taken together, we identify hypoxia and HIF-1 α activation as a potential aggravating factor in the development of NASH in a mechanism that involves caspase-1/inflammasome activation and cellular crosstalk.

Considering the pro-inflammatory role of EV (24-27), we also evaluated the content of EV. We could demonstrate the presence of cleaved and active caspase-1 in EV from hypoxic fat-laden hepatocytes and EV from serum of CDAA-fed mice exposed to IH. Collectively, these novel findings indicate that EV from hypoxic fat-laden hepatocytes induce pro-inflammatory and pro-fibrotic signals in Kupffer cells and LX-2 cells, respectively via a mechanism that involves activation of caspase-1 and inflammasomes.

In summary, this doctoral thesis identifies hypoxia as an aggravating factor in the pathogenesis of NASH. This aggravating effect is dependent on intact HIF-1 α signaling in hepatocytes and intercellular crosstalk between hepatocytes on the one hand and Kupffer cells and stellate cells on the other

hand. This crosstalk is dependent on extracellular vesicles released from hepatocytes and involves active caspase-1 and inflammasomes. We demonstrate that fat-laden hepatocytes are more susceptible to the damaging effects of hypoxia than non-steatotic hepatocytes and that hypoxia increases the extent of fat accumulation, inflammation and fibrosis both *in vitro* and *in vivo*. Further characterization of EV released under hypoxic conditions will allow the detailed delineation of their role in the promotion of steatosis, inflammation and fibrosis in the context of NAFLD and OSAS.

Some limitations of our studies include the possible contamination of the EV fraction with lipoproteins due to similarities in size and contamination, in the *in vivo* studies, with non-hepatic EV. Also, the small number of mice per group as well as the use of only 1 model of NASH are limitations. Future studies should include different models of NAFLD, e.g. high fat diet and also human studies, to confirm our findings. Also, the cargo of EV should be further analyzed for the presence of components, in addition to inflammasome components, that may modulate the response of Kupffer cells and stellate cells to EV. Our findings could have potential implications in the development of novel biomarkers that involve EV or selected components of EV, like caspase-1 and provide novel therapeutic targets for the treatment of NAFLD or liver damage in OSAS.

2. - PERSPECTIVES

The information generated in this thesis may contribute to a better understanding of intercellular communication in the pathogenesis of NAFLD and the development of novel (EV-based) biomarkers and therapies. It will also be interesting to perform similar studies in other liver diseases.

An exhaustive characterization of the content of EV isolated from *in vivo* and *in vitro* models of NASH, using genomic, proteomic and metabolomic techniques, will allow us to identify hypoxia-specific mechanisms related to hepatocellular damage that occurs in NASH. In addition, our experimental approach should be extended to clinical samples of patients with OSA and/or NASH, in order to generate translational data. These studies should include characterization of EV obtained from patients from blood or other bodily fluids such as urine or saliva. This non-invasive approach will allow the identification and validation of novel biomarkers and improve diagnosis and prediction of prognosis of NAFLD/NASH. Finally, non-pharmacological or pharmacological management of the levels of circulating EV can become a new therapeutic target in the context of NAFLD and OSAS.

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Appendices

English Summary

Background: Nonalcoholic fatty liver disease (NAFLD) is considered the most common liver disease worldwide. Transition from steatosis to non-alcoholic steatohepatitis (NASH) is a key issue in NAFLD. Observations in patients with obstructive sleep apnea syndrome (OSAS), which is characterized by occurrence of intermittent hypoxia (IH), suggest that hypoxia contributes to develop NASH. Among other mechanisms, release of extracellular vesicles (EV) by injured hepatocytes has been implicated in NAFLD progression. **Aim:** In this thesis we aimed to investigate the role of hypoxia condition in modulation of steatosis and liver injury in both in vitro and in vivo models of NAFLD. Also we evaluated the cellular crosstalk between hypoxic and steatotic hepatocytes and non-parenchymal cells through EV. **Methods:** Primary rat hepatocytes and hepatoma cell line HepG2 treated with free fatty acids (FFA) were subjected to chemically induced hypoxia (CH) using the hypoxia-inducible factor-1 alpha (HIF-1 α) stabilizer cobalt chloride (CoCl₂). Triglyceride (TG) content, oxidative stress, cell death rates, pro-inflammatory and pro-fibrotic cytokines, inflammasome components gene expression and protein levels of cleaved caspase-1 were assessed. Also, Kupffer cells (KC) and human stellate cells (LX-2) were treated with conditioned medium (CM) and EV from hypoxic fat-laden hepatocytes. The choline deficient L-amino acid diet (CDAA)-fed mice model used to assess the effects of IH on experimental NAFLD. **Results:** Hepatocytes exposed to FFA and CoCl₂ exhibited increased TG content and higher cell death rates as well as increased, oxidative stress and mRNA levels of pro-inflammatory, pro-fibrotic cytokines and inflammasome-components compared to non-treated hepatocytes. Protein levels of cleaved caspase-1 increased in CH-exposed hepatocytes and from EV-hepatocytes. CM and EV from hypoxic fat-laden hepatic cells evoked a pro-inflammatory and pro-fibrotic phenotype in KC and LX-2 respectively. Livers from CDAA-fed mice exposed to IH exhibited increased of steatosis, portal inflammation, fibrosis, mRNA levels of pro-inflammatory, pro-fibrotic and inflammasome genes as well as increased levels of cleaved caspase-1 that correlated with an increase of circulating EV-caspase-1. **Conclusion:** Our findings in both in vivo and in vitro models of NAFLD/NASH indicate that hypoxia may increase liver injury and promote disease progression through amplification of inflammatory and fibrotic signals including inflammasome/caspase-1 activation. Hypoxia also promotes the release of EV from hepatocytes contributing to cellular crosstalk with non-parenchymal cells by EV-caspase-1-related mechanisms. These results suggest EV and their content (caspase-1) as a potential novel biomarker in NAFLD and OSAS. Further characterization of EV released under hypoxic conditions will allow the detailed delineation of their role in the promotion of steatosis, inflammation and fibrosis in the context of NAFLD and OSAS.

Resumen

Antecedentes: El hígado graso no alcohólico (HGNA) es actualmente la enfermedad hepática más prevalente en todo el mundo. Los mecanismos que subyacen a la transición de la esteatosis a la esteatohepatitis no alcohólica (EHNA) no está del todo comprendida. Observaciones clínicas en pacientes con el síndrome de la apnea obstructiva del sueño (SAOS), caracterizada por la presencia de hipoxia intermitente (HI), sugieren que esta condición contribuye al desarrollo de EHNA. Recientemente, el rol de las vesículas extracelulares (EV) desde hepatocitos esteatóticos ha sido estudiado en la progresión del daño en el HGNA. **Objetivo general:** Investigar si modelos experimentales de EHNA son más susceptibles a los efectos de hipoxia, y si tal condición promueve el aumento de EV que señalizan a células no parenquimatosas. **Métodos:** Cultivo primario de hepatocitos y una línea celular HepG2 fueron tratados con ácidos grasos libres (FFA) y sometidos a hipoxia inducida químicamente (CH) usando cloruro de cobalto (CoCl₂). Se evaluó el contenido de triglicéridos (TG), el estrés oxidativo, muerte celular, la expresión génica de citoquinas pro-inflamatorias, pro-fibróticas, y de los componentes del inflamasoma, incluida la caspasa-1 escindida. Las células de Kupffer (KC) y las células estrelladas humanas (LX-2) se trataron con medio condicionado (CM) y con EV aisladas de hepatocitos. El modelo de ratones alimentados con L-aminoácido definido con deficiencia de colina (CDAA) se usó para evaluar los efectos de IH en HGNA experimental. **Resultados:** Los hepatocitos expuestos a FFA y CoCl₂ exhibieron un mayor contenido de TG, muerte celular, estrés oxidativo y un aumento en los niveles de la expresión génica de citoquinas proinflamatorias, pro-fibróticas y de los componentes de inflamasoma en comparación con los hepatocitos no tratados. Los niveles de caspasa-1 escindida aumentaron en los hepatocitos expuestos a CH, así como en las EV de los hepatocitos hipóxicos y esteatóticos. El CM y el mayor número de EV de las células hepáticas hipóxicas tratadas con FFA promovieron un fenotipo pro-inflamatorio y pro fibrótico en las KC y LX-2 respectivamente. Los hígados de ratones alimentados con CDAA expuestos a IH mostraron un aumento en la esteatosis, en la inflamación y fibrosis que se correlacionó con un aumento de las EV circulantes con cargo de caspasa-1. **Conclusión:** Nuestros hallazgos indican que los modelos experimentales de HGNA son más susceptibles a los efectos de la hipoxia que se correlacionan con el aumento de las EV. La hipoxia promovió señales inflamatorias y fibróticas, incluida la activación del inflamasoma/caspasa-1 en hepatocitos cargados de grasa y en nuestro modelo in vivo de EHNA, contribuyendo a la diafonía celular con células no parenquimatosas por EV-caspasa-1. Estos resultados proponen a las EV, y su contenido (caspasa-1), como un nuevo biomarcador potencial en HGNA y SAOS.

Nederlandse samenvatting

Achtergrond: Niet-alcoholische leververvetting (NAFLD) wordt wereldwijd beschouwd als de meest voorkomende leverziekte. Overgang van steatose naar niet-alcoholische steatohepatitis (NASH) is een belangrijk probleem bij NAFLD. Waarnemingen bij patiënten met obstructief slaapapneusyndroom (OSAS), dat wordt gekenmerkt door het optreden van intermitterende hypoxie (IH), suggereren dat hypoxie bijdraagt aan de ontwikkeling van NASH. Naast andere mechanismen is de afgifte van extracellulaire blaasjes (EV) door beschadigde hepatocyten betrokken bij NAFLD-progressie. **Doel:** In dit proefschrift wilden we de rol van hypoxie bij modulatie van steatose en leverbeschadiging in zowel in vitro als in vivo modellen van NAFLD onderzoeken. Ook evalueerden we de cellulaire samenwerking tussen hypoxische en steatotische hepatocyten en niet-parenchymale cellen via EV. **Methoden:** Primaire rattenhepatocyten en hepatoomcellijn HepG2 behandeld met vrije vetzuren (FFA) werden onderworpen aan chemisch geïnduceerde hypoxie (CH) met behulp van de hypoxie-induceerbare factor-1 alfa (HIF-1 α) stabilisator kobaltchloride (CoCl₂). Triglyceride (TG) -gehalte, oxidatieve stress, celsterftcijfers, pro-inflammatoire en pro-fibrotische cytokines, genexpressie van ontstekingscomponenten en eiwitniveaus van gesplitst caspase-1 werden beoordeeld. Ook werden Kupffer-cellen (KC) en humane stellaatcellen (LX-2) behandeld met geconditioneerd medium (CM) en EV van met hypoxische vet geladen hepatocyten. Het met choline-deficiënte L-aminozuurdieet (CDAA) gevoede muizenmodel dat werd gebruikt om de effecten van IH op experimentele NAFLD te beoordelen. **Resultaten:** Hepatocyten blootgesteld aan FFA en CoCl₂ vertoonden een verhoogd TG-gehalte en hogere celsterftcijfers, evenals verhoogde oxidatieve stress en mRNA-niveaus van pro-inflammatoire, pro-fibrotische cytokines en inflammasoomcomponenten in vergelijking met niet-behandelde hepatocyten. Eiwitniveaus van gesplitst caspase-1 namen toe in CH-blootgestelde hepatocyten en van EV-hepatocyten. CM en EV van met hypoxisch vet geladen levercellen wekten respectievelijk een pro-inflammatoire en pro-fibrotische fenotype op in KC en LX-2. Levers van CDAA-gevoede muizen die waren blootgesteld aan IH vertoonden verhoogde steatose, portale ontsteking, fibrose, mRNA-niveaus van pro-inflammatoire, pro-fibrotische en inflammasoomgenen, evenals verhoogde niveaus van gesplitst caspase-1 die correleerden met een toename van circulerende EV-caspase-1. **Conclusie:** Onze bevindingen in zowel in vivo als in vitro modellen van NAFLD/NASH geven aan dat hypoxie leverbeschadiging kan vergroten en ziekteprogressie kan bevorderen door versterking van inflammatoire en fibrotische signalen, waaronder de activering van inflammasoom/caspase-1. Hypoxie bevordert ook de afgifte van EV uit hepatocyten die bijdragen aan cellulaire overspraak met niet-parenchymale cellen door EV-caspase-1-gerelateerde mechanismen. Deze resultaten suggereren EV en hun inhoud (caspase-1) als een potentiële nieuwe biomarker in NAFLD en OSAS. Verdere

karakterisering van EV die vrijkomt onder hypoxische omstandigheden zal de gedetailleerde afbakening van hun rol bij de bevordering van steatose, ontsteking en fibrose in de context van NAFLD en OSAS mogelijk maken.

ACKNOWLEDGEMENTS

This doctoral thesis was supported by the Chilean government through Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1150327 and 1119145) and PhD fellowship from Comisión Nacional para la Investigación en Ciencia y Tecnología (CONICYT) and Vicerrectorate of Research and School of Medicine, PUC. Also, the support from the Abel Tasman Talent Program (ATTP) of the Graduate School of Medical Sciences and the University Medical Center Groningen (UMCG) from The Netherlands is gratefully acknowledged.

ABBREVIATIONS

OSAS: obstructive sleep apnea syndrome

IH: intermittent hypoxia

NAFLD: non-alcoholic fatty liver disease

NAFL: non-alcoholic fatty liver

NASH: non-alcoholic steatohepatitis

CDAA: choline-deficient amino acid-defined

NLRP3: NOD-like receptor Pyrin Domain Containing 3

GSDMD: gasdermin D

KC: Kupffer cells

TG: triglyceride

IL: interleukin

TNF- α : tumor necrosis factor-alpha

HIF-1 α : hypoxia inducible factor 1 alpha

EV: extracellular vesicles

FBS: fetal bovine serum

FFA: free fatty acids

CM: conditioned medium

AUF: Arbitrary units of fluorescence

PBS: phosphate-buffered saline

LDH: Lactate dehydrogenase

NTA: nanoparticle tracking analysis

ASC: Apoptosis-Associated Speck-Like Protein Containing CARD

SUPPLEMENTARY MATERIAL

Table S1: Sequences of primers and probes used for quantitative real time PCR

Gene		Primers	Specie
<i>NLRP3</i>	Sense	5'-GTG CGT GGG ACT GAA GCA T-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-CTG ACA ACA CGC AGA TGT GAG A-3'	
	Probe	5'-AGC AGC TGA CCA ACC AGA GTT TCT GCA-3'	
<i>ASC</i>	Sense	5'-AGC TGT GGC TAC TGC AAC CA-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-GAC CCT GGC AAT GAG TGC TT-3'	
	Probe	5'-ACA AAA TGT TCT GTT CTG GCT GTG CCC T-3'	
<i>Caspase-1</i>	Sense	5'-CGG AGT TTC CTA CTG AAT CTT TTA ACA-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-GAA AGA CAA GCC CAA GGT TAT CA-3'	
	Probe	5'-ACC ACT CCT TGT TTC TCT CCA CGG CA-3'	
<i>IL-18</i>	Sense	5'-ACC CTG CAG CTG GAG AGT GT-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-TTG ACT TCT ATC TTG TTGAAG ACA AAC C-3'	
	Probe	5'-CCC AAA CAA TAC CCA AAG AAG AAG ATG GAA AAG-3'	
<i>IL-6</i>	Sense	5'-CCG GAG AG GAG ACT TCA CAG A-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-AGA ATT GCC ATT GCA CAA CTC TT-3'	
	Probe	5'-ACC ACT TCA CAA GTC GGA GGC TTA ATT ACA-3'	
<i>TNF-α</i>	Sense	5'-GTA GCC CAC GTC GTA GCA AAC-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-AGT TGG TTG TCT TTG AGA TCC ATG-3'	
	Probe	5'-CGC TGG CTC AGC CAC TCC AGC-3'	
<i>iNOS</i>	Sense	5'-CTA TCT CCA TTC TAC TAC TAC CAG ATC GA-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-CCT GGG CCT CAG CTT CTC AT-3'	
	Probe	5'-CCC TGG AAG ACC CAC ATC TGG CAG-3'	
<i>IL-10</i>	Sense	5'-TGC AGG ACT TTA AGG GTT ACT TGG-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-CAG GGA ATT CAA ATG CTC CTT G-3'	
	Probe	5'-TCT CTG CCT GGG GCA TCA CTT CTA CCA-3'	
<i>Arg-1</i>	Sense	5'-AGC TGG GAA TTG GCA AAG TG-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-TCC AGT CCA TCA ACA TCA AAA CTC-3'	
	Probe	5'-AAT GGG CCT TTT CTT CCT TCC CAG CAG-3'	
<i>18S</i>	Sense	5'-CGG CTA CCA CAT CCA AGG A-3'	<i>Rattus norvegicus</i>
	Anti-sense	5'-CCA ATT ACA GGG CCT CGA AA-3'	
	Probe	5'-CGC GCA AAT TAC CCA CTC CCG A-3'	
<i>IL-18</i>	Sense	5'-ACA GAT GAA GTG CTC CTT CCA-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-GTC GGA GAT TCG TAG CTG GAT-3'	
	Probe	5'-CTC TGC CCT CTG GAT GGC GG-3'	
<i>TNF-α</i>	Sense	5'-CCC TGG TAT GAG CCC ATC TAT C-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-AAA GTA GAC CTG CCC AGA CTC G-3'	
	Probe	5'-ATC AAT CGG CCC GAC TAT CTC GAC TTT-3'	
<i>NLRP3</i>	Sense	5'-GGG ATT CGA AAC ACG TGC AT-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-CAG GAG AGA CCT TTA TGA GAA AGC A-3'	
	Probe	5'-ATC TGA ACC CCA CTT CGG CTC ATC TCT TT-3'	
<i>TGF-β1</i>	Sense	5'-GGC CCT GCC CCT ACA TTT-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-CCG GGT TAT GCT GGT TGT ACA-3'	
	Probe	5'-ACA CGC AGT ACA GCA AGG TCC TGG C-3'	
<i>CTGF</i>	Sense	5'-TGT GTG ACG AGC CCA AGG A-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-TCT GGG CCA AAC GTG TCT TC-3'	
	Probe	5'-CCT GCC CTC GCG GCT TAC CG-3'	
<i>α-SMA</i>	Sense	5'-GGG ACG ACA TGG AAA AGA TCT G-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-CAG GGT GGG ATG CTC TTC A-3'	
	Probe	5'-CAC TCT TTC TAC AAT GAG CTT CGT GTT GCC C-3'	
<i>Collagen-I</i>	Sense	5'-GGC CCA GAA GAA CTG GTA CAT C-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-CCG CCA TAC TCG AAC TGG AA-3'	
	Probe	5'-CCC CAA GGA CAA GAG GCA TGT CTG-3'	
<i>18S</i>	Sense	5'-CGG CTA CCA CAT CCA AGG A-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-CCA ATT ACA GGG CCT CGA AA-3'	
	Probe	5'-CGC GCA AAT TAC CCA CTC CCG A-3'	
<i>IFN-γ</i>	Sense	5'-ACT GTC GCC AGC AGC TAA AA-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-TAT TGC AGG CAG GAC AAC CA-3'	
<i>IL-18</i>	Sense	5'-AAG ATG GCT GCT GAA CCA GT-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-GAG GCC GAT TTC CTT GGT CA-3'	
<i>TIMP-1</i>	Sense	5'-CTT CTG GCA TCC TGT TGT TG-3'	<i>Homo Sapiens</i>
	Anti-sense	5'-GGT ATA AGG TGG TCT GGT TG-3'	
<i>TGF-β1</i>	Sense	5'-CTC CCG TGG CTT CTA GT GC-3'	<i>Mus musculus</i>
	Anti-sense	5'-GCC TTA GTT TGG ACA GGA TCT G-3'	
<i>CTGF</i>	Sense	5'-GGG CCT CTT CTG CGA TTT C-3'	<i>Mus musculus</i>
	Anti-sense	5'-ATC CAG GCA AGT GCA TTG GTA-3'	
<i>Collagen-I</i>	Sense	5'-GCT CCT CTT AGG GGC CAC T-3'	<i>Mus musculus</i>
	Anti-sense	5'-CCA CGT CTC ACC ATT GGG G-3'	
<i>α-SMA</i>	Sense	5'-GTC CCA GAC ATC AGG GAG TAA-3'	<i>Mus musculus</i>
	Anti-sense	5'-TCG GAT ACT TCA GCG TCA GGA-3'	
<i>TIMP-1</i>	Sense	5'-GCA ACT CGG ACC TGG TCA TAA-3'	<i>Mus musculus</i>
	Anti-sense	5'-ACT GTT CCT GAA CTC AAC T-3'	
<i>HIF-1α</i>	Sense	5'-AGG ATG AGT TCT GAA CGT CGA AA-3'	<i>Mus musculus</i>
	Anti-sense	5'-CTG TCT AGA CCA CCG GCA TC-3'	
<i>TNF-α</i>	Sense	5'-CCC TCA CAC TCA GAT CAT CTT CT-3'	<i>Mus musculus</i>
	Anti-sense	5'-GCT ACG ACG TGG GCT ACA G-3'	
<i>IL-18</i>	Sense	5'-ACT GTT CCT GAA CTC AAC T-3'	<i>Mus musculus</i>
	Anti-sense	5'-ATC TTT TGG GGT CCG TCA ACT-3'	
<i>IL-18</i>	Sense	5'-GAC TCT TGC GTC AAC TTC AAG G-3'	<i>Mus musculus</i>
	Anti-sense	5'-CAG GCT GTC TTT TGT CAA CGA-3'	
<i>NLRP3</i>	Sense	5'-ATT ACC CGC CCG AGA AAG G-3'	<i>Mus musculus</i>
	Anti-sense	5'-TCG CAG CAA AGA TCC ACA CAG-3'	
<i>Caspase-1</i>	Sense	5'-ACA AGG CAC GGG ACC TAT G-3'	<i>Mus musculus</i>
	Anti-sense	5'-TCC CAG TCA GTC CTG GAA ATG-3'	
<i>IL-6</i>	Sense	5'-TAG TCC TTC CTA CCC CAA TTT CC-3'	<i>Mus musculus</i>
	Anti-sense	5'-TTG GTC CTT AGC CAC TCC TTC-3'	
<i>IFN-γ</i>	Sense	5'-ATG AAC GCT ACA CAC TGC ATC-3'	<i>Mus musculus</i>
	Anti-sense	5'-CCA TCC TTT TGC CAG TTC CTC-3'	
<i>MCP-1</i>	Sense	5'-TTA AAA ACC TGG ATC GGA ACC AA-3'	<i>Mus musculus</i>
	Anti-sense	5'-GCA TTA GCT TCA GAT TTA CGG GT-3'	
<i>18S</i>	Sense	5'-CGG CTA CCA CAT CCA AGG A-3'	<i>Mus musculus</i>
	Anti-sense	5'-CCA ATT ACA GGG CCT CGA AA-3'	

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