Review

Sex difference in liver diseases: How preclinical models help to dissect the sex-related mechanisms sustaining NAFLD and hepatocellular carcinoma

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SUMMARY

Only a few preclinical findings are confirmed in the clinic, posing a critical issue for clinical development. Therefore, identifying the best preclinical models can help to dissect molecular and mechanistic insights into liver disease pathogenesis while being clinically relevant. In this context, the sex relevance of most preclinical models has been only partially considered. This is particularly significant in NAFLD and HCC, which have a higher prevalence in men when compared to pre-menopause women but not to those in post-menopausal status, suggesting a role for sex hormones in the pathogenesis of the diseases. This review gathers the sex-relevant findings and the available preclinical models focusing on both *in vitro* and *in vivo* studies and discusses the potential implications and perspectives of introducing the sex effect in the selection of the best preclinical model. This is a critical aspect that would help to tailor personalized therapies based on sex.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) covers a wide spectrum of pathological conditions, ranging from hepatic steatosis (NAFL) - which defines the condition of triglycerides (TG) accumulation in more than 5% of hepatocytes, not related to alcohol consumption - to the more severe and progressive form, namely non-alcoholic steatohepatitis (NASH). A recent meta-analysis has shown that about 59% of patients with fatty liver developed NASH.[1] The long-term clinical outcome of NASH is cirrhosis; however, ~50% of patients affected by hepatocellular carcinoma (HCC) may bypass cirrhosis demonstrating that NASH is an independent risk factor for cancer promotion.[2] Remarkably, the global incidence and prevalence of NAFLD and HCC are rapidly increasing.[3]

Consistent with the higher incidence and prevalence of NAFLD and HCC in males than in pre-menopausal females worldwide,[4] the hypothesis that a sex disparity exists and influences the onset and progression of liver disease has been postulated. In particular, the male sex seems to be more predisposed to developing hepatic fibrosis,[5] also associated with an increased probability of developing inflammation-driven HCC.[6] Not solely restricted to the incidence, the male predominance is also reflected by poor prognosis.[7] This gender bias has been attributed to the sexual dimorphism of the liver that differentially affects gene expression, lipid/glucose metabolism, immune response between male and female,[8,9] and subsequently susceptibility, progression, and outcomes of liver diseases. Increasing evidence demonstrates the implication of a plethora of different pathogenic determinants, including cytokines and hormones, that could be sex-related.[10] Notably, sex hormones exert a crucial role in the pathogenesis and development of NAFLD and HCC.[11] Indeed, NAFLD and HCC incidence markedly increases in postmenopausal women, when the levels of estrogens in the serum decrease.[12] It is therefore established that estrogens, androgens, and their cognate receptors can contribute to the sex differences in NAFLD and HCC onset and progression. In general, evidence suggests that androgens can promote steatosis[13,14] and tumor initiation and progression,[15] whereas the role of estrogens is mainly protective.[16] However, the underlying molecular mechanisms remain poorly understood, and gaining insights into the sex-dependent mechanisms of NAFLD progression and hepatocarcinogenesis could identify innovative molecular approaches and targets to combat liver diseases.

SEX-BASED *IN VITRO* MODELS OF NON-ALCOHOLIC FATTY LIVER DISEASE

A major challenge faced by translational research is related to the small percentage of preclinical findings confirmed in the clinic. Consequently, identifying the best models that can help to dissect the molecular and mechanistic insights into liver disease, taking into consideration the sex relevance, is crucial. In this session, we explore the available NAFLD preclinical *in vitro* models, with a particular focus on the pros and cons and their significance for the sex-related scenario.

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The more simplistic *in vitro* models are derived from cell lines cultured in two dimensions; independently of their source, most cell lines can be cultured at a large scale in a cost-efficient manner. However, most of the NAFLD-relevant studies did not specify the sex of the cells, although this aspect would be fundamental to highlight a possible sex-different response to the induction and progression of NAFLD and extrapolate clinically relevant information.

In the reviewed studies, NAFLD is generally induced by the administration of a mixture of unsaturated and saturated free-fatty acid (FFA) to the culture medium: a commonly used mix is composed by oleic acid (OA) added to palmitic (PA) or stearic acid (SA) in different proportions (accordingly to the different protocols), with the intent to cause a lipid overload into the hepatocytes hence inducing reactive oxygen species (ROS) accumulation and subsequent potential lipid peroxides generation.[17]

The cellular models that have been used to model NAFLD in the preclinical *in vitro* setting belong to three different classes: immortalized human hepatic cancer cells, primary human hepatocytes (PHH), and stem cells that can be differentiated into hepatocytes.

Human hepatocellular carcinoma cell lines grown as monolayers can be immortalized by genetic engineering or more frequently obtained from male and female human cancers. Hepatic cancer cells commonly used are HepG2 and Huh7, which are derived from male patients, and HepaRG, from a female biopsy: these cells have the advantage of a high proliferative capacity, phenotype stability and reproducibility, ready availability, ease of use and genetic manipulation, low cost of maintenance.[18,19] However, it is not entirely clear whether they retain a metabolic capacity resembling that of a normal hepatocyte, an aspect that may be key in understanding the lipid metabolic features of NAFLD-induced cells.[20] Moreover, the use of liver tumor cell lines to study NAFLD progression is not optimal as they may show an important deregulation of metabolic and molecular traits that could be crucial for NAFLD initiation and progression. Of the several hepatic tumor cell lines commercially available,[21] the most commonly used is the HepG2 cell line, which originated from the liver biopsy of a 15-year-old Caucasian male (Cellosaurus RRID: CVCL_0027, DepMap ID: ACH-000739). Notably, caution should be used with the HepG2 cell line since (i) has been mistakenly labeled as HCC instead of hepatoblastoma[22] and (ii) displays a weak or absent expression of the cytochrome P450 (CYP) superfamily,[23] which is involved in phase 1 xenobiotic oxidation in the liver and also represents a potential source of ROS.[24] HepG2 cells are principally used for drug safety and toxicity assays, molecular target screening,[25,26] and studies related to NAFLD disease and HCC biology.[25–27] This cell line displays all the main features of a human liver neoplastic transformation (e.g., enhanced protein levels of transferrin, α2-macroglobulin, and α-fetoprotein) and is negative for hepatitis virus.[28]

Among the male-derived cancer cell lines, Huh7 cells, derived from a well-differentiated HCC in a 57-year-old male (Cellosaurus RRID: CVCL_0336, DepMap ID: ACH-000480), have been rarely used in the context of NAFLD induction.[29,30]

On the other hand, the human hepatoma HepaRG cell line derived from the liver of a European female (age not reported) diagnosed with chronic hepatitis C and macronodular cirrhosis[31,32] (Cellosaurus RRID: CVCL_9720) is a female-relevant cellular model. These cells are phenotypically stable thus permitting long-term culture and represent a useful tool in the validation of drugs targeting in 3D organotypic human hepatic steatosis models,[33] preclinical drug screening and metabolism assays, and in studies of carcinogenesis and hepatitis B virus (HBV) infection.[34,35]

NAFLD induction using OA administration for a short-term pulse of 16 or 24 h was reported in different studies using HepG2,[36,37] with a dose-dependent effect associated with lipid peroxidation and apoptosis.[38] It has been shown that lipid accumulation caused by OA in HepG2 can be significantly reduced by treatment with exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist.[37] Although no female-derived cell lines were used in the study, a subsequent report demonstrated that exendin-4 is also effective in reducing lipid content and liver inflammation in female mice,[39] suggesting that, in this scenario, the observed phenotype is relevant for both sexes.

An innovative approach to induce steatosis consists of culturing HepG2 cells in a high-energy mimicking diet using OA and fructose for 24 h. This condition caused lipid accumulation in HepG2 cells with concomitant alterations in mitochondrial integrity, dynamics, and oxidative phosphorylation, suggesting the dominant role of mitochondria in the first hit of NAFLD progression.[40] Although these data were reported in HepG2 cells, it has been demonstrated that female rats show larger and more functional mitochondria than males pointing to sex hormones as relevant signals in the modulation of mitochondrial biogenesis and function.[41,42] In fact, estrogen treatment enhanced mitochondrial content and oxidative capacity in the liver of ovariectomized (OVX) rats (i.e., with circulating estrogen levels similar to those of post-menopausal women) and in HepG2 cells, while reducing hepatic lipid accumulation and oxidative stress, suggesting a protective role of estrogens in the liver pathology.[43] Similar results were also obtained in Huh7 cells exposed to OA and PA in which the estrogen treatment reduced lipid deposition, ROS production, and subsequent lipid peroxidation, a process reverted by the administration of an estrogen receptor antagonist.[44]

PHH derived from male and female human donors represent the closest model to study sex dimorphism due to their high similarity with the *in vivo* setting.[45] Although the PHH represent the gold standard short-term human *in vitro* steatotic liver model, they have major limitations as (i) the short propagation time when in culture, (ii) the high phenotypic instability, and (iii) the donor-to-donor variability.[18]

NAFLD induction is obtained by exposing PHH to PA either alone or in combination with OA, a stimulus that leads to lipid accumulation associated with endoplasmic reticulum stress[46] and profibrogenic phenotype.[47] Interestingly, PHH have been cultured in a 3D spheroid system to mimic an *in vivo* human hepatic steatosis[48,49]: a treatment with FFA and insulin at physiological and pathophysiological concentrations induced a steatotic phenotype and insulin resistance within 21 days.[50,51] In this context, it would be even more relevant to specify the sex of the PHH donors as it would further contribute to the investigation of sex differences in the induction and progression of steatosis. However, very few studies were conducted taking this important bias into account. In one of these, PHH obtained from steatotic male and female liver tissues showed no significant difference in the ability to reduce oxidative stress and lipid deposition when exposed to compounds that reduce fat deposition.[52]

Finally, in the last decade, the study of NAFLD induction and progression has benefited from the advent of models derived from human embryonic pluripotent stem cells (hESC) and human-induced pluripotent stem cells (hiPSC), which can be differentiated into hepatocyte-like cells (HLC). hESC derived from the inner cellular mass of a male or female pre-implanted embryo display pluripotency *in vitro* and *in vivo* and can be differentiated into tissues from all the three germ layers (endoderm, mesoderm, and ectoderm),[53] thus representing an ideal tool to model sex-related phenotypes in liver disease and progression thanks to their high culture stability and reproducibility together with the possibility to be expanded *in vitro*.[54,55] hiPSC have the advantage that can be obtained from any human somatic cells through the ectopic expression of transcription factors and exhibit unlimited self-renewal ability and ease of accessibility to donor tissues thus enhancing their versatility.[56,57] Moreover, HLC exhibit morphology, metabolism, and transcriptome profile similar to those of PHH with the advantage of a reduced functionality drift over cell culture passages,[58,59] thus representing an important resource for *in vitro* human liver disease processes investigation. Despite the aforementioned advantages, the use of HLC is limited by ethical restrictions that are different in different countries or by potential issues related to the lack of a standardized differentiation protocol that could lead to an incomplete hepatic differentiation.[19]

An important hESC-derived model to study NAFLD progression consists of a 48 h OA administration to HLC derived from male hESC WA01 cells (Cellosaurus RRID: CVCL_9771). This stimulus induces an increase in the intracellular lipid droplets (LD) content accompanied by Perilipin 2 (PLIN2) and Peroxisome Proliferator Activated Receptors (PPAR) pathway enhanced expression and activation, respectively. Moreover, Sinton and colleagues proposed a human-relevant model of hepatic steatosis that can be used for high-resolution analysis of metabolic function during NAFLD progression by exposing HLC derived from hESC female WA09 (Cellosaurus RRID: CVCL_9773) to lactate, pyruvate, and octanoate (LPO). In addition to the canonical macro-vesicular steatosis observed in HLC, LPO stimulation induces the disruption of the electron transport chain activity related to enhanced tricarboxylic acid (TCA) cycle anaplerosis, with the concomitant compensatory purine nucleotide cycle shunt leading to an overgeneration of fumarate.[60] On these premises, it would be interesting to compare male WA01 and female WA09 cell lines differentiated in HLC to (i) evaluate potential sex-dependent differences in steatosis etiology and progression and (ii) investigate whether there is a sexual dimorphism related to the oxidant and inflammatory response, thus shedding light on the role of estrogens in this context.

Alternatively, a 24 h OA administration to donor-derived hiPSC differentiated into HLC can be exploited to study NAFLD progression.[61] Taking the ethical issues aside, using different donors could be relatively straightforward to evaluate the sex- or estrogen-dependent differences in these models. However, HLC derived from hiPSC are mostly used to model the early-stage events of NAFLD. For instance, HLC differentiated from hiPSC derived from 2 male and 2 female donors with liver disease have been exposed to OA for several days, revealing induced lipid accumulation and a distinct steatosis signature for each stage of the liver disease of the donors. This phenotype was paralleled by significant differences in the transcriptome of the four representative models with the highly steatotic cells characterized by low expression of genes associated with gluconeogenesis, phospholipid and cholesterol biosynthesis together with the concomitant low expression of carnitine palmitoyl transferase 1A (CPT1A), the rate-limiting enzyme responsible for the transport of fatty acid (FA) derived acyl-CoA across the mitochondrial membrane, indicating a potential lower capacity of energy generation.[62] Although male and female donors were present in the study, the heterogeneity of the liver disease represented by the models makes it hard to conclude anything meaningful related to sex. Further investigations on additional patients and healthy donor liver specimens are necessary to untangle the contribution of sex hormones-related mechanisms in liver disease progression.

Overall, although many aspects of NAFLD can be recapitulated *in vitro*, models that take into consideration the genotype, age, and sex of the donors could have a key impact on the analyses of the mechanisms underlying this disease.

SEX-BASED *IN VITRO* MODELS OF HEPATOCELLULAR CARCINOMA

In light of the remarkable sex disparity and despite considerable efforts devoted over time, newly well-designed sex-related models are necessary for both basic and translational research to explore the role of sex in liver cancer. The ideal sex-based model should be reliable, highly reproducible, technically simple, and with a reduced cost. Moreover, the ideal model should recapitulate the key events observed during hepatocarcinogenesis and HCC progression in males and females in view of establishing effective personalized preventive/therapeutic strategies or unraveling novel sex-related prognostic/predictive markers. Existing data are principally centered on animal models and limited are those on the *in vitro* sex disparities relevant to humans.[63] Although the complexity of the *in vitro* models is considerably ameliorated in the last few years, a unique model able to reproduce the sex disparity in HCC does not exist. The currently employed sex-based *in vitro* models remain limited and inadequate with a weak translational value and need further technological improvements to be more reliable. The present subtask provides a state-of-the-art overview of the currently available sex-based *in vitro* models commonly applied for the study of HCC and summarizes their pros and cons.

The sex-based *in vitro* models of HCC are basically restricted to 2D cultured cell models. While being the most valuable cost-effective preclinical models routinely used in liver cancer research, the 2D cell cultures grown as monolayers do not recapitulate the intricated *in vivo* liver tumor microenvironment. Moreover, the adhesion to a rigid surface may influence the canonical cellular functions, and the risk of cross-contamination with other cell lines as well as the predisposition to genetic alterations occur after a long-term culturing.[64,65] However, no 3D sex-based model has been currently reported. The introduction of an *in vitro* 3D model that resembles the cell-microenvironment interplay may provide a more realistic preservation of the *in vivo* sex disparity thus filling the gap between 2D and animal models, although some limitations including high cost, laborious handling, and a reduced time of persistence in culture will need to be addressed.[66]

As for preclinical studies on NAFLD, the *in vitro* models currently available to elucidate the role of sex disparity in HCC onset and progression range from hepatic cancer cell lines to PHH and stem cell-derived models.

Liver cancer cell lines are the most popular model used since they extensively cover a large proportion of genetic and epigenetic alterations that distinguish the tumor of origin.[66] However, each of the commonly used tumor-derived human liver cell lines is originated from a single male or female donor. Therefore, in addition to the high donor-to-donor variability, they do not completely preserve the HCC biology and fail to reproduce the inter- and intra-tumor complexity of the liver environment.[67] This issue may be partially bypassed by selecting a panel of different male and female HCC cell lines.[63,68] Moreover, most liver cell cultures lack central hepatic markers and features and appear to be less differentiated when compared to PHH.[69] Not more than thirty liver cancer cell lines have been described and are currently available for HCC studies.[70] Among these, male HepG2 and female HepaRG cells emerge as the most engaged experimental models relevant for the exploration of sex disparity in HCC[35,71,72] thanks to their availability and multifaceted phenotype, although a restricted number of comparative studies between male and female cell lines has been published.[63] Indeed, in the majority of the studies relevant to HCC, authors usually did not specify the sex of the models enrolled, making it difficult to extrapolate the sex perspective of the study and the subsequent clinical application.

The majority of the cell lines recurrently adopted in liver cancer research are of male origin.[26,32,72–74] Hep3B cells represent an 8-year-old juvenile male HCC (Cellosaurus RRID: CVCL_0326, DepMap ID: ACH-000625); while C3A is a subline of the HepG2 cells, showing a morphology more hepatocyte-like when compared to the parental cells. In stark minority, among the female models, SNU-387 cells represent a grade IV/V pleomorphic HCC derived from a 41-year-old female (Cellosaurus RRID: CVCL_0250, DepMap ID: ACH-000478) while Hepa1-6 is a female murine hepatic cancer cell line originated from a spontaneous tumor grown in a C57BL/J mouse (Cellosaurus RRID: CVCL_0327).

Sex disparity has been extensively investigated *in vivo* while the *in vitro* experimental design is often deficient.[75,76] An innovative approach consists of oncogenic hepatocytes isolated from doxycycline K-ras^{V12}-induced transgenic zebrafish that have been used to demonstrate a male-dependent cortisol role in sustaining HCC sex discrepancy by heavily enhancing the infiltration of tumor-associated neutrophils (TAN) and tumor-associated macrophages (TAM) through the stimulation of the attractant transforming growth factor β 1 (TGF-β1). This male-biased mechanism was inhibited by estrogens in females. To note, zebrafish emerges as an ideal model for investigating the cortisol-induced effects since both humans and zebrafishes exploit cortisol as their main stress hormone whereas mice and rats mainly use corticosterone.[77,78]

Various studies employing different *in vitro* cellular models have proposed estrogens to have a protective role in mitigating HCC development.[79,80] Among these, a comparison between the female-derived liver cancer cell line SNU-387 and various male-derived tumor cells (Hep3B, HuH7, and HepG2.2.15) identified a novel mechanism in which estrogens, particularly estradiol (E2), transcriptionally activated miR-23a and p53 via estrogen receptor alpha (ERα) thus controlling apoptosis in liver cells.[63] Similarly, Guo et al. speculated the existence of a link between E2 and apoptosis in human liver cancer male HepG2 cells that may contribute to the unique sex disparity observed in HCC by increasing Foxo3a phosphorylation and inducing oxidative stress. This association was not observed in HepG2.2.15 cells, a cell clone derived from HepG2 and harboring the HBV (Cellosaurus RRID: CVCL_L855), and in LO2 cells that are normal hepatocytes derived from human embryos, thus delineating a cell-specific role of E2 in regulating the sex difference that characterizes HCC.[26] Moreover, by analyzing a panel of male HCC cell lines (HA22T, HuH-7, Hep3B, and HepG2), Huang's lab disclosed that ERα or ERβ may act as tumor suppressors in downregulating PPARγ expression in a ligand-dependent manner exclusively in Hep3B cells.[81] The murine liver female Hepa1–6 and male AML12 (isolated from the normal liver of a 3-month-old mouse) cell lines have been used to reinforce the concept that female resistance to tumor initiation and progression is hormone-dependent, as evidenced by the protective role exerted by prolactin mobilization in preventing HCC by hampering a tumor-promoting TNF receptor-associated factor (TRAP)-dependent innate immune response within hepatocytes.[82] Male HepG2 and HepG2.2.15 cell lines have also been used as an androgen receptor (AR) positive model to demonstrate that dihydrotestosterone (DHT)-induced AR activation enhanced the toll-like receptor 4 (TLR4) transcriptional activation and subsequent downstream signaling thus promoting proliferation, colony formation ability, migration, and invasion. Crucially, this phenomenon was not observed in the AR-negative Hepa1-6 cells. In contrast, E2 significantly down-regulated TLR4 expression in HepG2 and did not potentiate any of the aggressive features displayed by these AR-positive cells, thus suggesting that DHT-AR-TLR4 signaling is a critical mechanism underlying sex difference in HCC.[71] Importantly, a sex difference has been also described in the mechanism regulating the M2 polarization of macrophages within liver cancer. Specifically, it has been reported that E2 suppressed macrophage alternative activation and tumor progression by inhibiting the Jak1-Stat6 signaling pathway in female Hepa1-6 cells and in *in vivo* mouse models.[83] Murine H22 male hepatic carcinoma cells have been used to further corroborate the protective role of estrogen through the activation of ERα, which down-regulated NF-κB activity, and ultimately attenuated tumor growth and invasion, thus contributing to elucidate the sex-dependent molecular mechanisms in HCC.[73] Collectively, although some of the findings obtained in male models have been replicated in the female one, the relevance of sex hormones in liver diseases is confirmed at epidemiological and clinical levels. It is therefore possible that some findings derived from the male models could undermine a female-relevant mechanism. This aspect should be therefore considered prospectively when designing sex-relevant liver studies.

Liver cancer cell lines have also been used to evaluate the therapeutic and adverse effects of different pharmacological approaches. For instance, the cytotoxicity of three different commercial anti-cancer drugs (i.e., cisplatin, 5-fluorouracil, and doxorubicin) has been assessed in two human male-derived (Hep3B and SK-Hep1) and two female-derived (SNU387 and SNU878) liver cancer cell lines and a trend of doxorubicin-related dose-dependent cytotoxicity was observed exclusively in the male models,[84] suggesting that the effect of a chemical compound could be different based on the selected *in vitro* model and highlighting that sex is an essential consideration to take into account in drug screening and development.

PHH isolated from liver tissue have been principally used to elucidate the physiological liver function but are also a valid model for studying HCC initiation.[85,86] Further applications of PHH consist of (i) 3D culture approach as spheroidal aggregates able to retain the main hepatocyte functions for a longer time[87] and (ii) studies of biotransformation and toxicity, including those used to evaluate drug-induced liver injury.[88–91] Unfortunately, studies on sex differences have been rarely performed with these models due to the poor characterization of the male and female donors.[92]

Murine primary hepatocytes and primary HCC cells have been exploited to support the male predominance in HCC.[85] Primary hepatocytes were isolated by collagenase perfusion[93] from male and female mice while liver tumors from diethylnitrosamine (DEN)-induced HCC were mechanically digested to derive primary HCC cells.[93,94] Results showed that testosterone deprivation and estrogen supplementation were positively correlated with loss of cyclin E and functional G1/S checkpoint control, as well as with the induction of p53-and p21-mediated cell death of primary hepatocytes and HCC cells, thus strengthening the potent role of sex hormones in the sex difference of hepatocarcinogenesis.[85] Although several studies demonstrated that estrogens reduce liver cancer cell proliferation, some other studies demonstrate how this hormone can accelerate proliferation rate via either the canonical ERα axis activated during liver regeneration or the orphan nuclear receptor estrogen-related receptor γ (ERRγ)[95] in liver cancer cells.[96] Although more frequently employed to clarify HCC onset and biology, PHH have also been engaged in toxicity studies. Research in this field has been mainly achieved either *in vivo* with animal studies or *in vitro* with human immortalized cell lines.[97–99] The first attempt to reveal sex-related differences in terms of response to various hepatotoxic drugs was performed by Mennecozzi et al.[100] by comparing male and female (pre- and post-menopausal to include female age-correlated hormonal alterations) cryopreserved PHH pooled from twelve donors per group, an approach that has taken into consideration the individual genetic variability. Considering the remarkable genome dissimilarities between men and women, the authors assumed that cells may present singular sex-defined characters and revealed an overall greater sensitivity to hepatotoxicants in female primary hepatocytes.[100]

To note, several studies have used the same PHH derived from both male and female donors: namely, FT294 from 55 years old female with a neoplastic obstacle in bile conduct, FT296 from 76 years old male with liver metastasis from colon cancer, FT297 from 82 years old male with cholangiocarcinoma, FT288 from 77 years old male with hepatic metastasis, and FT289 from 51 years old female with HCC.[88,101,102] To untangle the role of sex disparity in HCC initiation and progression, the aforementioned PHH could be used as a model in which HCC could be induced, taking into consideration the role of sex-related molecular mechanisms and the role of hormone responsiveness.

Finally, an original model that could facilitate sex studies in HCC are the HLC derived from the differentiation of hESC or hiPSC, that offer an unlimited source of cells that can be cultured for a long time in the absence of loss functionality.[103,104] Unfortunately, an important limitation is represented by the lack of standard protocols useful for the differentiation of hESC and hiPSC into HCC cells and, to date, no relevant studies are exploring their potential application in sex disparity.

In summary, the present subtask notifies that the identification of the appropriate model to elucidate the impact of sex in HCC remains an essential step requiring detailed documentation based on the nature of the study. Indeed, several advantages and limitations have been proposed but no model is currently suited to recapitulate the complex heterogeneity of the *in vivo* liver tumor microenvironment.

Tables 1 and 2 and Figure 1 include a summary of the available preclinical *in vitro* models that could help to elucidate sex differences in liver diseases.

SEX-BASED *IN VIVO* MODELS OF NON-ALCOHOLIC FATTY LIVER DISEASE

Animal models of NAFLD have been recognized as useful tools in elucidating the molecular mechanisms and pathophysiology underlying the development of NAFLD. The experimental models of NAFLD that recapitulate different phases and aspects of this hepatic disease comprise diet-induced, chemical, and genetic models or their combination. Importantly, each animal model is characterized by its advantages and disadvantages and no single animal model thoroughly encompasses the full spectrum of human NAFLD.[105,106] Although a recent mouse sex-based liver metabolic computational model, applied to identify the most relevant sex-dependent metabolic pathways involved in the initial steps of NAFLD, reached the conclusion that female and male livers are two metabolically distinct organs,[107] most animal studies performed so far focused and still focus on only one sex. The lack of sex-based comparison studies and the choice of male as the predominant experimental model significantly limit research, not only in the field of NAFLD,[108] frequently leading to inconclusive results and observations. Moreover, animal models of NAFLD that specifically reflect sex differences observed in humans have not been established yet.

Diet-induced animal models of non-alcoholic fatty liver disease

To date, the most relevant animal models that improved our understanding of sex disparity in NAFLD and examined sex differences in this spectrum of hepatic diseases are those diet-induced. These experimental models are based on various types of diet such as high fat diet (HFD), high glucose, sucrose, fructose, choline- and methionine-deficient diet (CMD), choline-deficient L-amino-defined diet (CDAA) and high cholesterol diet (HCD).[105,109]

Feeding a CMD diet results as one of the best-described dietary models of NAFLD, which also sheds light on some important aspects of sex differences in NAFLD development. This nutritional regimen leads to a rapid accumulation of TG in hepatocytes.[110] NAFL development in the CMD model is the consequence of a block in the synthesis of phosphatidylcholine (PC), an essential component of very low-density lipoproteins (VLDL).[111] From a translational point of view, feeding a CMD diet represents a frequently employed animal model presenting close pathological and biochemical similarities to NASH.[112] As reported by Kirsch et al.,[113] feeding a CMD diet for 4 weeks induced steatosis that resulted more severe in male than female rats. It has been proposed that biochemical mechanisms underlying the PC biosynthesis in the liver of rats may be responsible for the sex dimorphism observed in males and females.[113] PC synthesis occurs via two pathways: the first one

Table 1. A summary of sex-relevant *in vitro* models of NAFLD

NAFLD	Cell and method of induction	Observations	Reference
Male	OA administration for a short-term pulse of 16 or 24 h in HepG2 cell line	Lipid droplets accumulation associated with lipid peroxidation and apoptosis. These evidences were significantly reduced by treatment with exendin-4.	Alkhatatbeh et al.,[36] Khalifa et al.,[37] Cui et al.[38]
	High-energy diet mimicking condition with OA and fructose for 24 h in HepG2 cell line	In parallel to lipid accumulation, observed alterations in mitochondrial integrity, dynamics, and oxidative phosphorylation. Estrogen treatment reduced lipid accumulation and enhanced mitochondrial content and oxidative capacity.	Swapna Sasi et al.,[40] Galmés-Pascual et al.[43]
	Huh7 were induced by OA and PA administration for 48 h	Lipid content and ROS production were reduced by estrogen, a process reverted by the administration of an estrogen receptor antagonist.	Farruggio et al.[44]
	Treatment with OA for several days in hiPSC derived donors	Different steatotic features related to the liver disease stage of the donors.	Graffmann et al.[62]
	Treatment with LPO for 48 h in hESC WA09	Steatotic phenotype with compromission of mitochondria activity and concomitant development of a compensatory purine nucleotide cycle shunt leading to the excess generation of fumarate.	Sinton et al.[60]
	Treatment with OA for several days in hiPSC derived donors	Different steatotic features related to the liver disease stage of the donors.	Graffmann et al.[62]

involves the direct incorporation of preformed choline into phosphatidyl compounds while the second one is dependent upon the stepwise methylation of phosphatidylethanolamine (PE) by S-adenosyl methionine (SAM).[114] Female rats were reported to be more dependent than males on the stepwise methylation of PE.[115–117] This difference was attributable to the estrogen status that enhanced females' capacity to produce PC via the ER-dependent regulation of phosphatidylethanolamine N-methyltransferase (PEMT) pathway.[117–119] In fact, female rats fed a choline-deficient, but not methionine-deficient diet, developed less NAFL than male rats.[115] However, less hepatic steatosis was consistently observed in female rats subjected to the CMD experimental protocol, strongly suggesting the contribution of other mechanisms, allowing methionine-dependent pathways of PC synthesis in females.[113] Accordingly, a deficient choline intake has been significantly associated with increased fibrosis in postmenopausal women with NAFLD.[120] The study performed by Kirsch et al.[113] also analyzed the effect of the CMD diet on mice. Male C57/BL6 mice fed for 4 weeks developed less steatosis than rats but demonstrated strong necroinflammation, early evidence of fibrosis, and histological features of NASH. A more severe NAFL in male mice fed a CMD diet for 2 weeks has been also confirmed by Lee et al.[121] To explain the mechanism responsible for the protection of female mice from the CMD-induced NAFLD, the authors concentrated their attention on the involvement of sex-specific metabolic interactions in the crosstalk between peripheral tissues, adipose tissue, and liver.[122] In fact, they demonstrated that the CMD diet increased the expression of brown adipocyte markers and genes related to mitochondrial FFA β-oxidation in gonadal white adipose tissue (gWAT) of female mice, by involving the fibroblast growth factor 21 (FGF21), which has been reported to increase the appearance of brown-like adipocytes in subcutaneous WAT.[123] In contrast, in another study,[124] the histological examination of livers from CMD-fed mice reported that the degree of lipid content and necroinflammation was comparable in males and females, as well as serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and liver TG levels. Remarkably, ovariectomy and feeding CMD diet containing tamoxifen had no protective effect in females.[124] Although the CMD protocol induces the onset of a NASH histological phenotype in a short time frame and mimics the human disease, its main disadvantage is the lack of any other metabolic features, that are frequently observed in human NAFLD, such as obesity or peripheral insulin resistance. Moreover, the animals lose rather than gain weight in the CMD model.[105,106]

Feeding an HFD, that encompasses multiple regimens in which fat content varies between 45 and 75% kcal,[105] has been reported to develop insulin resistance with marked panlobular steatosis, inflammation, and fibrosis.[109] A chronic exposure to an HFD mirrors the metabolic profile of human NASH in mice, by inducing hyperglycaemia and insulin resistance.[125] However, it should be underlined that the results obtained with HFD may vary and are influenced by the composition of the diet and the rodent strain.[109] In this regard, in a recent study, which involved 9 inbred mouse strains fed with either a chow diet or HFD, Bachmann et al.[126] highlighted the importance of taking into consideration the genetic background and sex before drawing conclusions from HFD feeding experiments.[126] Considering the important effect of fructose on glucose and lipid metabolism, the HFD diet can be implemented with fructose either added to the drinking water or incorporated in the diet to mimic the high-fructose corn syrup particularly abundant in the last years in the Western diet (WD). Interestingly, non-genetically modified mice fed a high-fat, high-fructose (HF/HF) diet develop obesity, increased hepatic oxidative stress, and a NASH-like phenotype with significant fibrosis.[127] In this regard, Ganz et al.[128] concluded that male mice fed an HFD supplemented with fructose and sucrose for 6

Table 2. A summary of sex-relevant *in vitro* models of HCC

HCC	Cancer cell lines/cells derived from	Observations	Reference
Male	Doxycycline K-ras^{V12} induced transgenic zebrafish	Cortisol increased leukocyte infiltration in male hepatocytes. This male-biased mechanism was inhibited by estrogens in females.	Egan et al.[77]
	HepG2	Role of E2 in controlling apoptosis in liver cells.	Guo et al.[26]
	Hep3B	ERα or ERβ may act as tumor suppressors in downregulating PPARγ expression in a ligand-dependent manner.	Lin et al.[81]
	HepG2 and HepG2.2.15	E2 significantly down-regulated TLR4 expression, which transcription is favourited by dihydrotestosterone (DHT)-induced AR activation.	Han et al.[71]
	DEN-induced murine HCC	Estrogen suppressed hepatocyte cell cycle markers, upregulated p53 and reduced viability of HCC cells.	Pok et al.[85]
	H22	Estrogen attenuated tumor growth and invasion down-regulating NF-κB activity.	Xu et al.[73]
Female	Hepa1-6 cells	E2 suppressed macrophage alternative activation and tumor progression by inhibiting the Jak1-Stat6 signaling pathway.	Yang et al.[83]
	DEN-induced murine HCC	Testosterone increased expression of cyclin D1, cyclin E and reduced p53 and p21.	Pok et al.[85]
	Twelve donors	Female primary hepatocytes are more sensitive to hepatotoxic drugs.	Mennecozzi et al.[100]

and 16 weeks developed significant insulin resistance, NASH and displayed inflammasome components upregulation and activation (such as ASC, Aim2, NLRC4), as well as an increase in caspase-1 activity and IL-1β protein, after a long-term 16-week feeding. On the contrary, female mice presented only elevated TG levels, and high lipid content but did not show the activation of the inflammasome pathway or fibrosis.[128]

The analysis of sex differences in NAFL in a large cohort of diverse inbred strains of mice, known as the Hybrid Mouse Diversity Panel (HMDP),[129] fed a high-fat high-sucrose (HF/HS) diet (16.8% kcal proteins, 51.4% kcal carbohydrates, and 31.8% kcal fat) for 8 weeks revealed that males had higher hepatic TG levels and developed more severe steatosis compared to females. Remarkably, this observation regarded most out of 100 female and 113 male mice strains analyzed.[130] In the same study, it has been proposed that the difference in hepatic TG accumulation between females and males might be in part explained by striking differences in body fat distribution, plasma high-density lipoprotein (HDL) and genetic regulation.[130] In fact, a multi-omics integrative study performed on HMDP mice to uncover networks/pathways underlying NAFLD pathogenesis and elucidate sex differences in animal models of NAFLD revealed that lipid pathways, insulin signaling and inflammation markers in the adipose and liver tissue are associated with NAFLD in male HMDP.[131,132] Furthermore, liver pyruvate kinase (L-PK or Pklr) has been proposed as a candidate gene driving NAFLD in mice fed an HF/HS diet, as high levels of L-PK were strongly associated with NAFLD severity.[131] L-PK has been reported to act in a male-specific manner in the development of liver steatosis and fibrosis. In fact, while L-PK overexpression exacerbated liver steatosis in male mice, female mice overexpressing L-PK remained unaffected.[133] Furthermore, the analysis of more than 100 inbred strains of male and female mice fed a 8-week HF/HS diet (32% kcal from fat and 25% kcal from sucrose) demonstrated that insulin resistance, estimated by homeostasis model assessment for insulin resistance (HOMA-IR), was higher in males versus their female counterparts.[134]

Several studies investigated whether levels of manganese superoxide dismutase (MnSOD), a mitochondrial protein involved in ROS scavenging,[135] might contribute to increased oxidative stress, disease progression and reflect sex differences in rodent models of NAFLD. Levels of MnSOD were found reduced in the liver of male mice fed an HDF and male leptin deficient (Lep^{ob}/Lep^{ob}) ob/ob mice.[136] On the other hand, in female mice fed a Paigen diet for 14 and 24 weeks (17% fat, 1.25% cholesterol and 0.5% sodium cholate), that leads to the development of NASH, increased liver TG, inflammation, and fibrosis,[137] MnSOD protein levels were comparable to controls.[136] However, the levels of MnSOD resulted significantly reduced in female animals fed a CMD for 14 weeks.[136] Similar data have been reported also in male mice fed a CMD diet,[138] indicating that NAFLD induced by this experimental model is associated with low MnSOD in both males and females. Worthy of comment are also the results derived from studies which analyzed farnesoid X receptor (FXR) knockout (KO) mice fed a WD (21.2% fat, 34% sucrose, and 0.2% cholesterol, w/w).[139] FXR, a member of the nuclear hormone receptor superfamily, plays an essential role in regulating bile acid, lipid, and glucose homeostasis. Both male and female FXR$^{-/-}$ mice spontaneously develop liver tumors and have increased susceptibility to colitis, cholestasis, and colon cancer.[140] Feeding a WD in FXR$^{-/-}$ mice caused NAFL, which was more severe in males than females. Moreover, the expression levels of proinflammatory genes were higher in male than female WD-fed FXR$^{-/-}$ mice.[139] Using the WD diet in male and female C57BL/6J mice at 5, 10, and 15 months of age,[141] Hasegawa et al.[141] investigated its impact on the metabolome and the gut microbiota. Male and female mice significantly differed when considering their serum, urine metabolomes, and fecal and cecal microbiota.[141]
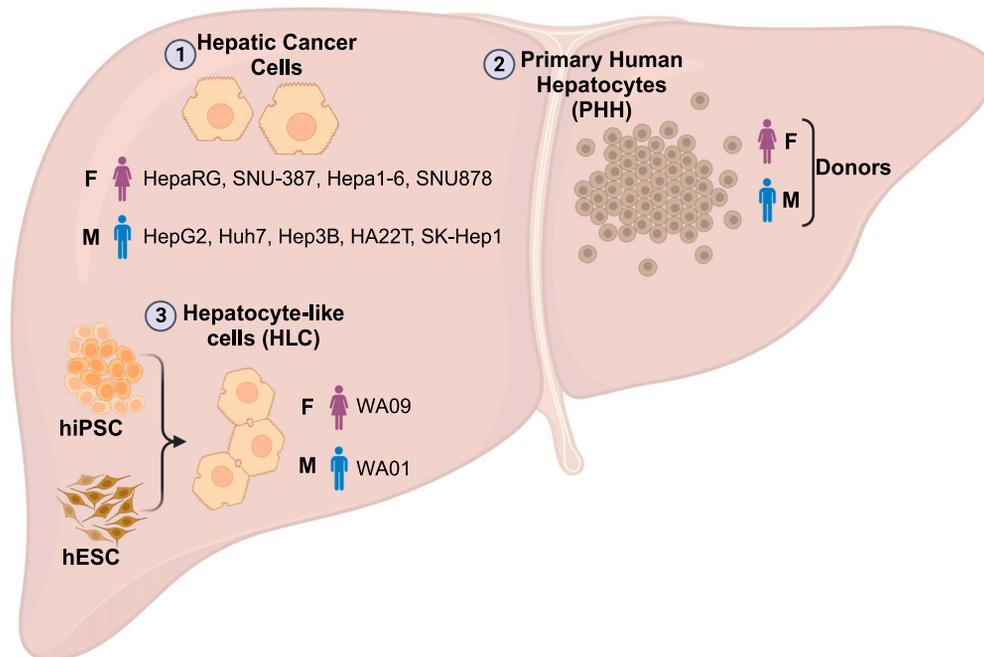
Figure 1. Overview of sex-relevant *in vitro* models of NAFLD and HCC
The figure shows a schematic representation of the pre-clinical models of NAFLD and HCC and the available male and female cellular sources. The main models utilized are based on (1) hepatic cancer cell lines from male (HepG2, Huh7, Hep3B, HA22T, SK-Hep1) or female origin (HepaRG, SNU-387, Hepa1-6, SNU878); (2) primary human hepatocytes (PHH) derived from male or female donors and (3) hepatocyte-like cells (HLC), derived from human-induced pluripotent stem cells (hiPSC) or human embryonic pluripotent stem cells (hESC, for example male WA01 and female WA09 hESC). The Figure was created using Biorender.

Although female rodents were also reported to be protected from the adverse metabolic effects induced by a 60% high-fructose diet, such as increased plasma TG, hyperinsulinemia, and hypertension, this protection may be lost after ovariectomy, suggesting the contribution of female sex hormones in the defense against the effects of a fructose diet.[142] Ovariectomy was shown to increase HOMA-IR in female mice on both chow and HF/HS diet, demonstrating that estrogens provide insulin-sensitizing effects in female mice[134] and, in accordance with previous studies, demonstrating that ovariectomy accelerates the development of insulin resistance in rodents.[143] Nonetheless, a differential response to the gonadectomy has been reported in different strains of mice fed an HF/HS diet. As described by Norheim et al.,[130] while C57BL/6J and DBA/2J tended to increase hepatic TG, C3H/HeJ showed decreased hepatic TG levels.[130]

Although numerous animal studies suggested that male sex may be a risk factor for the development of NAFLD, there is some evidence indicating that females might be at higher risk for NAFLD development. Administration of a 10% (w/v) fructose solution in drinking water in Sprague-Dawley rats for two weeks induced hepatic steatosis and hypertriglyceridemia in both sexes, while hyperinsulinemia only developed in female rats.[144] Female C57BL/6J mice chronically exposed to 30% fructose solution for 16 weeks to induce steatosis resulted more susceptible to NAFLD. Although NAFL was almost similar between male and female mice, inflammation was more pronounced in livers of female mice. This difference was associated with the alterations in the regulation of the adiponectin-AMP-activated protein kinase (AMPK)-plasminogen activator inhibitor 1 (PAI-1) signaling cascade in the liver.[145] Higher lipid percentage area, liver TG content, and more pronounced hepatic steatosis have been also reported in female mice[146] fed a "cafeteria" diet (CAF), a model of obesity-inducing diet consisting of highly palatable, energy-dense human junk foods.[147,148]

In summary, the studies regarding sex differences in nutritional animal models of NAFLD seem a bit conflicting so far. Several animal models of NAFLD were characterized by severe steatosis and NASH in males, recapitulating the main clinical feature of human NAFLD. On the other hand, also the opposite observation has been reported. Moreover, some animal models of NAFLD showed no clear sex differences. Generally, studies reporting male predominance were based on CMD- and HFD-nutritional approaches. In contrast, a higher female susceptibility to NAFLD was observed in high-fructose or CAF models.

Table 3 includes a summary of the reviewed studies employing nutritional approaches to elucidate sex differences in animal models of NAFLD.

Genetic animal models of non-alcoholic fatty liver disease

With the advancement in genetic engineering, it has been possible to create various genetic animal models of NAFLD that can mimic some characteristics of human NAFLD. Among the most frequently used genetically modified, transgenic or KO mice, in the context of NAFLD we can include ob/ob mice carrying a spontaneous mutation in the leptin gene and are leptin deficient, therefore resulting in an inability to detect

Table 3. A summary of sex-relevant *in vivo* nutritional models of NAFLD

NAFLD	Species/Strain/Model	Observations	Reference
Dietary models	Wistar, Long-Evans, Sprague-Dawley rats fed a CMD diet for 4 weeks C57/BL6 mice fed a CMD diet for 4 weeks	Greater steatosis, higher liver lipid content, and ALT levels in male rats. N ecroinflammation. Histological features of NASH in male mice when compared to females and Wistar rats.	Kirsch et al.[113]
	C57BL/6N mice fed a CMD diet for 2 weeks	Overt steatosis in males.	Lee et al.[121]
	C57BL/6J mice fed a CMD diet or CMD diet supplemented with tamoxifen (0.01%) for 4 weeks	CMD-induced steatohepatitis comparable in male and female mice. No protective effect in CMD-induced steatohepatitis by ovariectomy or tamoxifen treatment.	Kashireddy et al.[124]
	C57Bl/6 wild-type mice fed an HFD supplemented with fructose and sucrose for 6 and 16 weeks	Steatohepatitis and inflammasome activation in males at 16 weeks, high fibrosis markers in males. Steatosis without inflammation in females at 16 weeks.	Ganz et al.[128]
	A cohort of inbred mice strains-Hybrid Mouse Diversity Panel fed a high-fat high-sucrose (HF/HS) diet for 8 weeks	Higher hepatic TG and more severe steatosis in male mice.	Norheim et al.[130]
	C57BL/6 mice and FXR$^{(-/-)}$ mice fed a Western diet	Severe steatohepatitis in FXR$^{(-/-)}$ male mice.	Jena et al.[139]
	C57BL/6J mice exposed to a 30% fructose solution for 16 weeks	Higher susceptibility to NAFLD in female mice.	Spruss et al.[145]
	Young female (FCaf) and male (MCaf) Swiss mice fed a cafeteria diet for 14 weeks	More extensive steatosis in FCaf than in MCaf.	Gasparin et al.[146]

satiety.[112] As a consequence, ob/ob mice become hyperphagic, extremely obese and display insulin resistance and hyperinsulinemia. Although ob/ob mice develop mild to severe steatosis, this genetic model requires a second hit, such as a chemical challenge lipopolysaccharide, carbon tetrachloride (CCl_4), thioacetamide (TAA) administration or a nutritional regimen (CMD diet and HFD), to trigger the progression to NASH.[149]

Another frequently applied model is represented by db/db mice, which have a natural mutation in the leptin receptor gene, causing a phenotype similar to that observed in the ob/ob mice. An analogous mutation in rats is known as Lepr^{fa}/Lepr^{fa} (fa/fa, Zucker rats) and shows similar histologic and metabolic characteristics to those reported in ob/ob and db/db mice. Similarly, fa/fa rats require an additional stimulus to develop NASH.[105,150] However, it should be considered that the abovementioned mutations are very rare in humans.

Among the plethora of the genetic models available in mice, it is worth mentioning the sterol regulatory-element binding protein (SREBP)-1c transgenic mice, characterized by the overexpression of the lipogenic transcription factor SREBP-1c in the fat tissue, the mice KO for PPAR-α, a key regulator of genes involved in peroxisomal and mitochondrial FA oxidation systems, and the phosphatase and tensin homologue-(PTEN) deficient mice, that spontaneously develop steatosis, NASH and HCC.[105,150]

Notably, Schiffrin et al.[151] analyzed three genetic mouse models of NAFLD: ob/ob, lipodystrophic fat-specific (*Pparg*F$^{Δ/Δ}$) and whole-body PPARγ-null mice (*Pparg*$^{Δ/Δ}$ mice), totally deprived of adipose tissue. PPARγ is a nuclear receptor that promotes adipocyte differentiation and maturation.[152] While no apparent dimorphism was observed in ob/ob and *Pparg*F$^{Δ/Δ}$ mice, a clear sex dimorphism was reported in *Pparg*$^{Δ/Δ}$ mice with female presenting macro- and micro-vesicular hepatic steatosis throughout their entire life. Remarkably, sex differences were lost in gonadectomized *Pparg*$^{Δ/Δ}$ mice.[151] An increased visceral adipose tissue (VAT) storage, adipose tissue inflammation, micro-vesicular steatosis were reported in young male low-density lipoprotein receptor-deficient (Ldlr−/−) Leiden (Ldlr−/−.Leiden) mice fed an HFD for 18 weeks.[153]

Recently, Dungubat et al.[154] examined whether sex differences were present in Tsumura Suzuki obese diabetes (TSOD) and db/db mice fed a normal diet and could be age-dependent. TSOD mice represent a polygenic model of metabolic syndrome and spontaneously develop diabetes mellitus, obesity, glucosuria, hyperglycemia, and hyperinsulinemia.[155] The levels of serum ALT and AST were significantly higher in male than female mice at the age of 3 months in both strains. While conspicuous hepatic steatosis was observed in male db/db mice at the age of 3 months, intralobular inflammation tended to be more severe in male TSOD mice. The fibrotic area was significantly higher in male than female mice of both strains at the age of 3 months. For all the parameters analyzed, no significant sex differences were observed at 9 months in both strains.[154]

Among transgenic mouse models with a sex-specific metabolic phenotype, we can also include *Akr1d1*$^{-/-}$ mice generated on a C57BL/6 background.[156] The enzyme Aldo-Keto Reductase Family 1 Member D1 (Akr1d1) has a central role in the regulation of glucocorticoid and bile acid, recognized as potent regulators of metabolism and energy balance.[157] Male Akr1d1$^{-/-}$ mice challenged with an HFD (60% kcal from fat) for 20 weeks were partially protected against diet-induced hypertriglyceridemia but displayed glucose intolerance or insulin resistance.[156]

Table 4. A summary of sex-relevant *in vivo* genetic models of NAFLD

NAFLD	Species/Strain/Model	Observations	Reference
Genetic models	Ob/ob, lipodystrophic fat-specific (PparγF$^{\Delta/\Delta}$), whole-body PPAR-null (Pparγ$^{\Delta/\Delta}$) mice	No apparent dimorphism in ob/ob and lipodystrophic fat-specific (*Pparg*F$^{\Delta/\Delta}$) mice; higher TG storage and hepatic steatosis in female Pparg$^{\Delta/\Delta}$ mice.	Schiffrin et al.[151]
	Young Ldlr$^{-/-}$Leiden mice fed an HFD for 18 weeks	Increased VAT storage, adipose tissue inflammation, microvesicular steatosis in males.	Jacobs et al.[153]
	Tsumura Suzuki obese diabetes (TSOD) and db/db mice fed a normal diet	ALT and AST levels, hepatic steatosis and the fibrotic area significantly higher in males at the age of 3 months no significant sex differences at 9 months.	Dungubat et al.[154]
	Akr1d1$^{-/-}$ mice fed a standard diet until 10 weeks of age and then transferred to HFD for 20 weeks	Partial protection against diet-induced hypertriglyceridemia but not glucose intolerance or insulin resistance in male Akr1d1$^{-/-}$ mice.	Gathercole et al.[156]
	Cyp3a-null mice fed an HFD fed an HFD for 8 weeks	Increased hepatic TG and macrovescicular steatosis in Cyp3a-null male mice.	Kumar et al.[161]
	Cyp2b-null mice fed a CDAHFD for 8 weeks	Higher TG levels in Cyp2b-null males lower susceptibility to the development of NAFLD and less inflammation in females.	Heintz et al.[162]
	IL22ra1$^{-/-}$ mice on C57BL/6N background fed an HFD for 30 weeks	Severe liver injury, inflammation and fibrosis in female mice with NAFLD.	Abdelnabi et al.[165]
	Hepatocyte-specific Pten-deficient mice (C57BL/6J background) at 40 weeks and 76 weeks	Attenuated hepatic steatosis and inflammation in females compared to males.	Anezaki et al.[166]
	p62 and Nrf2 DKO mice fed a normal chow diet from 8 to 48 weeks of age	Later onset of steatohepatitis, milder hepatic inflammation and fibrosis in female mice.	Watahiki et al.[167]

Several studies concentrated on the role of different CYP in NAFLD. For example, hepatic steatosis was associated with decreased hepatic CYP3A activity,[158] that accounts for approximately 30–40% of the total CYP content in human adult liver and small intestine.[159] Interestingly, Finn et al.[160] reported that the inactivation of the hepatic cytochrome P450 system through the conditional deletion of P450 oxidoreductase (POR) induced hepatic steatosis.[160] Analysis of sex differences in Cyp3a-null mice fed an HFD for 8 weeks demonstrated that Cyp3a-null female mice were protected from the effects of an HFD, gained less weight and had improved glucose tolerance. On the contrary, Cyp3a-null male mice showed increased liver TG and macro-vesicular steatosis.[161] Using Cyp2b-null mice[162] fed a choline-deficient, L-amino acid-defined high fat diet (CDAHFD) for 8 weeks, it has been assessed whether the role of Cyp2b in NAFLD differs based on sex. The CDAA and CMD diets share choline deficiency, but in the CDAA diet proteins are substituted with a mixture of L-amino acids. Animals on this nutritional regimen develop a severe degree of NASH.[105] Also in this model, in contrast to males, CDAHFD-fed Cyp2b-null females were less susceptible to diet-induced NAFLD. Among the sex-dependent parameters, serum glucose, glucose tolerance, liver injury markers, and TG accumulation were identified. CDAHFD-fed Cyp2b-null female mice presented lower serum ALT and AST levels and a reduced inflammation.[163]

Another model that elucidated sex differences in NAFLD included the lack of interleukin-22 (IL-22), a pleiotropic cytokine that combines both inflammatory and protective effects during injury and repair in various tissues, including the liver.[164] Abdelnabi et al.[165] demonstrated exacerbated liver damage, NASH-related inflammation, and apoptosis in female IL22ra1$^{-/-}$ fed an HFD (40% kcal fat) for 30 weeks. Furthermore, the lack of IL22-receptor signaling promoted the progression of NASH-related fibrosis.[165] Using hepatocyte-specific Pten-deficient mice, Anezaki et al.[166] demonstrated that NAFL and inflammation were attenuated in females when compared to males.[166] Sex differences in the NASH phenotypes, resembling clinical features of human NASH, stemmed also from studies involving p62/Sqstm1 and nuclear factor E2-related factor-2 (Nrf2) double-knockout (DKO) mice. In fact, DKO female mice were characterized by a later onset of steatohepatitis, milder inflammatory responses, and fibrosis when compared to DKO males.[167] It has been suggested that, in female DKO mice, a low-grade inflammatory hit under conditions of high estradiol levels, with an effect similar to leptin,[168] could be attributable to less severe features of NASH.[167]

Table 4 includes a summary of the sex-based studies regarding genetic models of NAFLD.

SEX-BASED *IN VIVO* MODELS OF HEPATOCELLULAR CARCINOMA
Non-alcoholic fatty liver disease-induced models of hepatocellular carcinoma

Representative and reliable animal models of NAFLD-induced HCC are poor and liver cancer development is slow and rare.[169] Employing a NASH-HCC mice model consisting of a combination of chemical and dietary protocols - streptozotocin (STZ) - HFD, Fujii et al.[170]

demonstrated that all male mice subjected to the STZ-HFD regimen developed HCC in agreement with the reported male predominance in the HCC human development. On the contrary, in female mice treated with STZ–HFD, HCC development was not detected.[170] Interesting results stemmed also from studies in which female and male C57BL/6N mice were fed a choline-supplemented, high *trans*-fat, fructose, and cholesterol diet (CS-HFFC) or a choline-deficient, high *trans*-fat, fructose, and cholesterol diet (CD-HFFC). While choline deficiency led to lean NASH-HCC, choline supplementation allowed hepatocarcinogenesis in the context of obese NASH-HCC. At the endpoint (64 weeks of age), Hymel et al.[171] observed that tumor progression was faster in males. None of the female lean mice fed a CD-HFFC diet developed HCC (only dysplastic nodules were reported), while 50% of female obese mice fed a CS-HFFC diet developed HCC at the endpoint. Lean and obese males developed HCC at 64 weeks with similar penetrance (53%).[171]

Regarding HCC developed in genetic models of NAFLD, it has been reported that the incidence and the maximum size of liver tumors were significantly smaller in Pten-deficient female mice than males. Among the mechanisms underlying sex differences in this model, the authors described decreased ROS levels with increased antioxidant gene expression and decreased proinflammatory cytokine production in female mice.[166] Through the establishment of a mouse strain derived from outbred ddN colony, named fatty liver Shionogi (FLS), which develops a spontaneous progressive, severe hepatic steatosis without obesity and diabetes[172] and spontaneous hepatocellular adenoma (HCA) and/or carcinoma, it has been reported that HCC incidence was higher in males at the age of 16 months.[173] While at 16 months the liver tumors were found in 73% of male mice, HCC was not observed in all female mice examined at this time point. Only at 20–24 months of age, both HCA and HCC were present in 21% of female mice, suggesting also an earlier occurrence of liver cancer in male mice.[173] Like humans, female DIAMOND (diet-induced animal model of non-alcoholic fatty liver disease) mice, which develop NASH leading to HCC during Western diet (WD) feeding,[174] demonstrated lower susceptibility and lower incidence of NAFLD-associated HCC, coupled with distinct anatomical deposition of fat.[175] Male mice showed a significantly higher incidence of HCC (100% in males and 36% in females). While reversal of a WD to a chow diet (CD) at 36 weeks of WD feeding (corrective diet) caused a 60% inhibition of HCC development in males, a complete rescue from HCC was observed in females.[175]

Apart from NAFLD-associated animal models of HCC, among the experimental animal models of hepatocarcinogenesis that contributed to characterize the molecular mechanisms underlying HCC development and progression, we can also include chemically induced, genetically engineered mouse models, transgenic and KO mice, xenograft mouse models and patients derived xenografts (PDX).[176] However, none of them has been considered as an "ideal" one for all HCC research purposes.[177] Sex-based outcomes for HCC extend to other mammals and a sex disparity in HCC incidence similar to that observed in human patients has been described in several experimental models of hepatocarcinogenesis. In fact, the incidence of spontaneous and carcinogen-induced hepatic tumors is much lower in female rats and mice than in males.[178]

Chemically-induced and dietary models of hepatocellular carcinoma

Regarding the chemically-induced models, Nakatani et al.[179] demonstrated that, after a long-term repeated administration of the chemical carcinogen DEN, HCC developed in 100% of the male and in 30% of female mice, highlighting a differential response between both sexes. In the same study, the authors also demonstrated that such sex-related difference in HCC development and progression could be due to the inhibitory effect of estrogens and the stimulating effect of androgens on hepatocarcinogenesis. Indeed, orchidectomy significantly reduced the incidence of HCC in DEN-treated mice.[179] Nonetheless, the mechanisms responsible for this sex disparity and the estrogen-mediated anticarcinogenic activity remained unclear.

Sex disparity in HCC formation has been also described by Xie et al.,[180] who utilized streptozotocin-HFD in a murine model to investigate the relationship between gut microbiota and NASH-HCC development. 100% of STZ-primed neonatal male mice fed with HFD developed HCC at the time of 20 weeks. However, the percentage of HCC-bearing female mice was 12.5%. Indeed, only 1 out of 8 female mice developed liver tumors and the number of tumors in the single female was significantly lower than those found in male ones, thus highlighting a clear sex disparity in HCC development.[180]

To elucidate the mechanisms underlying sex disparity in HCC development, Naugler et al.[181] hypothesized that the inflammatory response could play a pivotal role in sex-related hepatocarcinogenesis. In fact, inflammation has been widely recognized as a major contributing factor for carcinogenesis.[182] In this context, Interleukin-6 (IL-6) is a multifunctional cytokine, whose serum concentration resulted increased during chronic liver inflammation including alcoholic hepatitis, HBV and hepatitis C virus (HCV) infections, the principal causes of chronic hepatitis[183] and NASH, well-known conditions that may lead to HCC development.[184] Moreover, patients with HCC display IL-6 serum levels significantly higher compared to liver cirrhosis group and healthy controls.[185] Naugler et al.[181] used a DEN-induced model, which is known to cause HCC in 100% of male mice but only in 10–30% of female littermates.[181] It has been reported that IL-6 is fundamental for liver parenchyma regeneration occurring during compensatory proliferation in DEN-induced hepatocarcinogenesis.[186] To investigate whether the sex bias in IL-6 production is responsible for the sex difference in HCC development, Naugler et al.[181] utilized a DEN-induced model of hepatocarcinogenesis in male and female mice KO for IL-6 (IL-6$^{-/-}$). While all male WT mice developed HCC, liver tumors were present in only 13% of female WT mice. Of note, a marked reduction in HCC incidence was observed in IL-6$^{-/-}$ males, whereas no difference was observed between WT and IL-6$^{-/-}$ females. Based on these premises, the authors investigated sex effects on DEN-induced IL-6 production. DEN administration resulted in an increased circulating IL-6. Moreover, the authors demonstrated that estrogens suppress IL-6 production leading to the inhibition of chemically induced liver carcinogenesis, suggesting a DEN-induced hepatocarcinogenesis dependency from an inflammatory response, triggered by hepatocyte necrosis, that leads to the production of IL-6.[181] Although the pathogenesis of HCC in abovementioned mouse model differs from human HCC, DEN-induced liver tumors have a histology and genetic signature similar to human HCC with poor prognosis.[187]

Genetic animal models of hepatocellular carcinoma

The sex-related disparity in HCC development has been also described in genetic murine models of hepatocarcinogenesis. Using an H-ras12V transgenic HCC murine model in a BCF1 (C57BL/6J/CBA/J) background, generated with H-ras12V under the control of the mouse albumin enhancer/promoter,[188] it has been demonstrated that HCC was prevalent in male H-ras12V transgenic mice. RAS-activated mutations have been found in all human tumors, and the frequency of *RAS* mutations is the highest among the genes that are correlated with human cancers,[189] highlighting how sex-dependent responses to deregulated Ras protein may contribute to this bias. Santoni Rugiu et al.[190] showed similar results using a genetic mouse model in which the hepatic co-expression of c-myc and TGF-α led to a major enhancement of HCC development. Both c-myc/TGF-α and c-myc transgenic females displayed longer latency and lower tumor incidence compared to males, even though with similar pathological changes, including the formation of HCC, which were absent in TGF-α single-transgenic females. While dysplasia developed at the same time in animals of both sexes, the males demonstrated a higher frequency of preneoplastic and neoplastic lesions. Although the tumors appeared later and with lower incidence in c-myc/TGF-α and c-myc females, it is worth emphasizing that the sequence of events, including the formation of malignant tumors, was essentially the same of the male mice. Notably, Takagi et al.[191] have shown that sex hormones exert a strong inhibition on the neoplastic process in the TGF-α transgenic female mice, resulting in approximately a 10-fold reduction of tumor incidence and absence of malignant tumors compared to males, suggesting that TGF-α-induced neoplastic events in the liver are more dependent from sex hormones than those caused by c-myc.

Using another transgenic mouse model of hepatocarcinogenesis characterized by an aberrant expression of miR-221 in the liver, Callegari et al.[192] demonstrated that while overexpression of miR-221 caused a spontaneous HCC development in the 50% of male mice, females did not develop any tumor. Furthermore, transgenic mice showed a higher susceptibility to HCC formation when treated with DEN, but still showing sex differences. Indeed, at 6 months, all male animals treated with the carcinogen developed multiple and large tumors compared to control mice. On the other hand, in females treated with DEN, liver tumors were visible and appreciable only at 9 months of age.[192]

Regarding viral infections, numerous evidence reported that HBV infection plays a pivotal role in HCC development. To investigate the consequences of the integration of HBV genes in the mice genome, Wang et al.[193] introduced the HBsAg and HBx genes in the same locus of p21 mainly in the liver tissues, leading to HCC development. Between the ages of 15 and 24 months, 8 out of 15 male mice p21HBsAg/+ heterozygotes (53.3%) and 8 out of 11 p21HBsAg/HBsAg homozygotes (72.7%) developed liver tumors. On the contrary, female mice did not display tumor development at the same age. Of note, at 18 months both male and female p21-HBx transgenic mice developed HCC. Finally, Moriya K et al.[194] investigated the penetrance of HCC in C21 and C49, two lines of HCV core gene transgenic mice.[194] The incidence of tumor formation in the male C21 and C49 mice was significantly higher than that of the controls, in contrast with a very low incidence in female transgenic mice, and in agreement with the epidemiological data indicating that men chronically infected with HCV are more likely to develop HCC than women similarly infected.[195]

Table 5 recapitulates the results of sex-based *in vivo* models of HCC included in this review.

Although several animal models reported NAFLD occurrence predominantly in males when compared to females, recapitulating the main clinical features of human NAFLD, opposite results or no evident sex differences were also described. In contrast, sex-based *in vivo* models of HCC, in the presence of NAFLD background or in other models of hepatocarcinogenesis, have clearly demonstrated HCC prevalence in males. In fact, the results reported in this section highlighted that all the experimental animal models, employed to unveil the mechanisms underlying HCC development and progression, were characterized by sex disparity like that observed in human HCC. The summary of the *in vivo* experimental models of hepatocarcinogenesis indicating the prevalence of NAFLD and HCC in males or females is reported Figure 2.

DISCUSSION AND OPEN QUESTIONS

Similar to other non-reproductive diseases, sex has undoubtedly a profound impact on NAFLD and HCC incidence in humans with a male bias in the global incidence. Male and female development diverges due to the profound impact of sexual chromosomes and the contribution of sex steroid hormones. These organizational and cell-intrinsic effects define sex differences by impacting on chromatin organization, metabolism, cell cycle regulation, immunity, and response to disease.[9,196]

Genetic and epigenetic aspects

One of the most fundamental drivers of sex differences lies within the nucleus of each cell, namely the XX (female) and XY (male) sex chromosome pairs. Women with one X chromosome have higher body weight and enhanced probability of developing metabolic disease than women with two X chromosomes. This type of genetic aberration is responsible for Turner syndrome in female and increases the risk of NAFLD and cirrhosis.[197] Moreover, Wiese et al.[198] demonstrated, by using four Core Genotypes mice (XX and XY mice with ovaries and XX and XY mice with testes), that the presence of Y chromosome controls the FA metabolism in hypercholesterolemic mice regardless of the presence of ovaries or testes and is responsible for cellular response to statin treatment.[198] Finally, the genetic background and chromosomal alterations influence the predisposition to the onset of a liver disease. For example, the I148M variant of the patatin such as phospholipase domain containing 3 (PNPLA3) gene on chromosome 22 confers increased risk for NAFLD and is predominant in women.[199] Conversely, the dysregulation of some specific genes on the Y chromosome, including RNA-binding motif gene on the Y chromosome and testis-specific protein Y-encoded (RBMY), regulates the AR activity and contributes to male predominance of HCC.[200] In addition to genetic factors, epigenetic mechanisms are also responsible for the sex differences observed in liver diseases. The main epigenetic modification related to the X-linked chromosome is DNA methylation that covers an important role in patients with NAFLD.[201–203] In fact, women presented a higher

Table 5. A summary of sex-relevant *in vivo* models of HCC

HCC	Species/Strain/Model	Observations	Reference
NAFLD-induced models	C57BL/6J mice subjected to STZ–HFD protocol	HCC presence in all male mice; no HCC development in female mice treated with STZ–HFD and male mice treated with STZ alone.	Fujii et al.[170]
	C57BL/6N mice fed a choline supplemented, high *trans*-fat, fructose and cholesterol diet (CS-HFFC) or a choline deficient, high *trans*-fat, fructose and cholesterol diet (CD-HFFC)	Faster tumor progression in males and slower in females in the context of lean NASH-HCC compared to obese NASH-HCC.	Hymel et al.[171]
	Hepatocyte-specific Pten-deficient mice (C57BL/6J background) at 40 weeks and 76 weeks	Lower HCC incidence and smaller HCC size in females.	Anezaki et al.[166]
	Fatty liver Shionogi (FLS) mice	Higher HCC incidence in male mice.	Soga et al.,[172] Soga et al.[173]
	DIAMOND mice fed a Western diet (WD) for 48–60 weeks	HCC incidence in 100% of males and 36% of females.	Mirshahi et al.[175]
Chemically- induced and dietary models	B6C3F(1) mice, treated with DEN (0.05 mg/mouse) every 2 weeks throughout the experimental period. At 6 weeks of age, mice were subjected to orchidectomy	100% of HCC incidence in males; 30% of HCC incidence in females with extensive chromosomal damage.	Nakatani et al.[179]
	C57BL/6J mice subjected to a single subcutaneous injection of 200 μg STZ and after 4 weeks fed with HFD for 16 weeks	100% of HCC in male mice, 12.5% of HCC in female mice.	Xie et al.[180]
	C57BL/6 WT and IL-6$^{-/-}$ mice injected intraperitoneally with DEN (25 mg/kg) at 15 days of age	HCC present in 100% of male WT and 13% of WT female mice, a marked reduction in HCC incidence in IL-6$^{-/-}$ males, no difference between WT and IL-6$^{-/-}$ females.	Naugler et al.[181]
Genetic models	Double transgenic mice overexpressing c-myc and TGF-α in the liver (Alb-c-myc/MT-TGF-α mice)	100% HCC in males and of 30% in females Alb-c-myc/MT-TGF-α mice.	Santoni-Rugiu et al.[190]
	B6D2F2 (C57BL/6J x DBA/2J) Transgenic (TG) mice overexpressing miR-221 in the liver	A spontaneous HCC development in 50% of male mice, no liver tumors in females.	Callegari et al.[192]
	H-ras12V transgenic mice in a BCF1 background	HCC prevalent in males.	Wang et al.[193]
	Transgenic mice carrying HBsAg or HBx in the p21 locus in a C57BL/6 background	53.3% p21HBsAg/+ heterozygotes males and 72.7% of p21HBsAg/HBsAg homozygotes male mice developed liver tumors; no HCC development in female mice; no difference in HCC development at 18 months in p21-HBx transgenic mice.	Wang et al.[193]
	Transgenic that carrying the HCV core gene in a C57BL/6 background	26-31% of HCC incidence in male transgenic mice; low incidence in females.	Moriya et al.[194]

methylation profile in the X chromosome with respect to men, which results in hepatic gene expression changes and subsequent lower cholesterol and triglycerides levels, in accordance with different metabolic activities.[204] Moreover, treatment with low-dose aspirin reduced body weight and hepatic lipid accumulation in female, but not in male, offspring mice with maternal over-nutrition as a high-risk model of obesity and NAFLD. In particular, the authors observed that aspirin reduced Wnt-signaling activity via hypo-methylation of the APC gene which explains its anti-proliferative effect interacting with beta-catenin only in female mice.[205]

Sex hormones

The fact that HCC is a sexually dimorphic tumor type in both rodents and humans is clearly driven by sex hormones. Indeed, besides their role in reproduction and sexual development, androgens and estrogens influence the risk of developing liver diseases and more generally
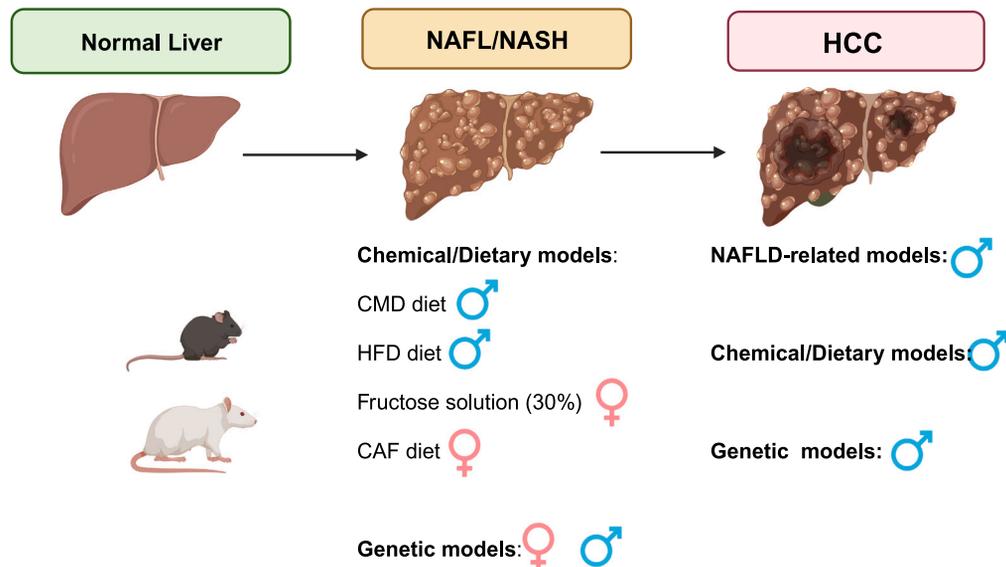
Figure 2. Overview of sex-relevant animal models of NAFLD and HCC
The Figure indicates the prevalence of NAFLD and HCC in *in vivo* male models compared to female models. The figure was created using Biorender.

metabolic diseases.[206] In particular, estrogens reduce the susceptibility to steatosis development in liver cells of female mice fed an HFD subjected to ovariectomy.[207] Moreover, the deletion of ERα in Western-type diet fed female and male mice reduced the ability of isolated hepatocytes to internalize high-density lipoprotein (HDL), although female mice displayed increased cholesterol and HDL serum levels, thus underlying an ERα-independent sex difference.[208]

A protective role of estrogens, which contribute to the sex dimorphism in HCC occurrence, has been confirmed in several studies. While the administration of estrogens suppressed chemically induced hepatocarcinogenesis in males,[209] ovariectomy had a promoting effect on tumorigenesis[210] and females lost their protection from HCC in the absence of ERα.[185] Although the molecular mechanisms responsible for sex-specific differences in HCC remain to be explored in depth, it has been hypothesized that the effects of estrogens and androgens on HCC development may be dependent on transcription factors Foxa1 and Foxa2.[211] Overwhelming evidence suggests that sex hormones may also affect mechanisms with a crucial role in HCC development and progression, such as metabolisms, oxidative stress, or tissue repair.[212]

Immune aspects

Sex disparities in liver disease cannot be elucidated without understanding the adaptive immune response of the liver tissue. Relative to the tissue-specific immune landscape, male and female present several differences in innate and adaptive immune response, since both chromosome-related genetic and epigenetic cues together with the impact of sex hormones can shape these functions. Indeed, immunological sex differences are present throughout life, whereas others are only apparent after puberty or before reproductive senescence.[212] Macrophages infiltration in adipose tissue of HFD-fed male is associated with enhanced inflammation and motility *in vitro* when compared to female-derived macrophages.[213] In addition, cytokine production can also be different during NAFLD progression: male-derived macrophages produce mainly pro-inflammatory cytokines whereas female-derived those with anti-inflammatory capacity.[214] Along this line, Scotland et al.[215] demonstrated that female mice have an immunological repertoire that facilitates sensing and elimination of pathogens, when compared to the male counterpart.[215] Overall, innate immune response in male mice promotes and sustains liver inflammation and subsequent disease, whereas the innate response of female liver cells seems to be dampened by estrogens which can impact cytokine production[216] not only on hepatocytes but also on Kupffer cells.[181] Adult females exhibit more robust cell-mediated and humoral immune responses to antigenic challenges.[212] ERs are expressed by many types of immune cells, including T cells, B cells, macrophages, neutrophils, natural killer (NK) cells[217] and estrogen increases the size of regulatory T_{reg}-cell population, reduces pro-inflammatory cytokine secretion, and increases the anti-inflammatory activity of neutrophils.[218] Among chromosome X-linked proteins with immune competent functions and roles in the inflammatory response, Toll-like receptors (TLRs), CD40 ligand, as well as the proteins associated with nuclear factor kB (NF-kB) signaling pathway are included.[219] Although one of the two X chromosomes in females is randomly inactivated by methylation, about 15% of X-linked genes escape inactivation to some degree.[220] Recently, it has been reported that distinct patterns of the immune response, regarding the ratio of CD4+/CD8+ T cells, Th1/Th2/Th17 cells, NKT/NK cells, M1/M2 macrophages, and T cell phenotypes in males and females are correlated with the progression or inhibition of NAFLD-associated HCC.[175] Moreover, distinct patterns of inflammatory cytokines and chemokines were detected in males and females during HCC progression.[175] Indeed, longitudinal studies elucidated the sex predisposition of males to NAFLD-associated HCC, suggesting that female mice were able to modulate aging-associated inflammatory cytokines compared to males.[175] Among the mechanisms responsible for the sex disparity in HCC, inflammatory response with the crucial role of

inflammatory cytokines has been also extensively studied.[218] Experimental evidence from rodent studies employing chemically induced models of hepatocarcinogenesis has provided mechanistic insight into the role of the inflammatory mediators. Naugler et al.[181] proposed that the estrogen-mediated inhibition of IL-6 production reduces HCC risk in females and loss of IL-6 neutralizes the sex disparity in hepato-carcinogenesis in mice.

Sex bias in HCC may be also attributed to environmental exposures (exposome), such as dietary patterns related to obesity, smoking, alcohol intake, and socio-economic status, which differ among individuals according to their gender and have been recognized as relevant risk factors for hepatocarcinogenesis. Although the fact that diet strongly influences gut microbiota composition and gut dysbiosis is involved in hepatocarcinogenesis, human studies unveiling the role of the gut microbiota in HCC are missing. Ponziani et al.[221] showed that Bacter-oides and Ruminococcaceae are more abundant in HCC when compared to cirrhosis without HCC. Moreover, the gut microbiota profile associated with HCC in patients with cirrhotic is characterized by increased Escherichia Coli fecal counts.[222]

CONCLUSIONS

The liver has been defined as an important sexually dimorphic organ. Population-based studies provided compelling evidence for a gender disparity in human liver pathologies, NAFLD and HCC. A distinctive feature in the epidemiology of these pathologies is the striking male inci-dence and prevalence when compared to pre-menopausal females.[4] Sex hormones have been shown to contribute to this sex bias and the protective effects of estrogen has been reported.[9,223]

The present review focused on the available sex-based comparison animal studies and *in vitro* approaches in the context of NAFLD and HCC. The analysis of collected data indicates that sex-based outcomes for NAFLD and HCC extend also to other mammals, revealing a higher susceptibility for males in rodent studies. However, it should be underlined that, although animal models undoubtedly provided a useful tool for the study of the pathogenesis of NAFLD and HCC, a limited number of sex-based comparison approaches exists. Most studies conducted so far in the field of NAFLD and HCC have been performed in male animals whereas the female sex is still highly underrepresented. Moreover, sex is often confused with gender making even harder for researchers that want to study this clinically relevant area of research to gain useful information. Therefore, our review will help researchers to have an overview of the models available to study the underlying mechanisms of sex differences in NAFLD and HCC. Data presented in our article emphasize the importance of considering sex in the design of experimental protocols in order to reach conclusive results.

Such approach is fundamental not only for the elucidation of mechanisms implicated in NAFLD and HCC pathogenesis, but also for un-derstanding and improvement of existing and novel treatment strategies. This is a critical aspect as gaining information on the sex differences underlying the mechanisms of liver disease would have a tremendous impact in sex-specific personalized therapies. This clinically relevant goal will be achieved by taking into consideration sex and reproductive status in the study design, not only in the clinical trials but also in the pre-clinical setting.

AUTHOR CONTRIBUTIONS

MAK, AP, and AM conceived and supervised the study. AS, NL, MS, and MAK wrote the original draft of the article. All the authors reviewed the prepared article.

DECLARATION OF INTERESTS

All the authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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