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Hepatic sexual dimorphism — implications for non-alcoholic fatty liver disease

Philippe Lefebvre^{1,†} and Bart Staels¹,

¹Univ. Lille, INSERM, CHU Lille, Institut Pasteur de Lille, U1011 - EGID, F-59000 Lille, France.

†philippe-claude.lefebvre@inserm.fr

Abstract

The liver is often thought of as a single functional unit, but both its structural and functional architecture make it highly multivalent and adaptable. In any given physiological situation, the liver can maintain metabolic homeostasis, conduct appropriate inflammatory responses, carry out endobiotic and xenobiotic transformation and synthesis reactions, as well as store and release multiple bioactive molecules. Moreover, the liver is a very resilient organ. This resilience means that chronic liver diseases can go unnoticed for decades, yet culminate in life-threatening clinical complications once the liver is no longer able to compensate for them. Non-alcoholic fatty liver disease (NAFLD) predisposes individuals to cirrhosis and increases liver-related and cardiovascular disease-related mortality. This Review discusses the accumulating evidence of sexual dimorphism in NAFLD, which is currently rarely considered in preclinical and clinical studies. Increased awareness of hepatic sexual dimorphism could lead to improved understanding of the biological processes that are dysregulated in NAFLD, to the identification of relevant therapeutic targets and to improved risk stratification of patients with NAFLD undergoing therapeutic intervention.

[H1] Introduction

The search for treatments for chronic metabolic diseases and other human ailments has often highlighted the difficulty of conducting drug trials in fully representative populations, as patient selection and randomization often introduces bias¹. The ability to define the factors that drive a patient's response to treatment is the key concept of precision medicine, which aims to treat only sensitive subpopulations. Surprisingly, essential biological differences between male and female individuals are rarely considered during preclinical² and clinical³ studies. This lack of consideration is a cause for concern because sex is a strong determinant of disease prevalence and outcomes in many fields, including cardiology⁴, diabetology⁵, oncology⁶, pulmonology⁷ and, of specific interest to this Review, hepatology. Nevertheless, the vast majority of therapeutic guidelines are optimized for a standard 70 kg (white) male patient. The consequences of overlooking sex as a biological variable (reviewed in depth elsewhere⁸) are often deleterious for both sexes and can result in inadequate initiation and choice of treatments. Given the existence of physiological sexual dimorphism in many metabolic processes, it is not surprising that pathogenic events can affect metabolically active organs

differently in male and female individuals. As such, the liver responds differently in men and women during the progression of chronic liver diseases, including non-alcoholic fatty liver disease (NAFLD)⁹.

First described in the early 1980s, NAFLD is a condition defined by histological abnormalities in the liver similar to those encountered in patients with alcoholism¹⁰. The pathogenesis of NAFLD is driven by metabolic dysfunction in the context of a fatty liver and thus this disease has a high prevalence in obese and diabetic patients []. When fat accumulation becomes chronic, hepatic steatosis (also termed fatty liver or non-alcoholic fatty liver (NAFL)) progresses to non-alcoholic steatohepatitis (NASH) and fibrosis¹¹. NASH was recognized as an important liver disease in the late 1990s¹² and the pathogenic events leading to NASH and end-stage liver fibrosis (cirrhosis) were subsequently shown to result from a 'multi-hit' mechanism in which steatosis paves the way for additional hepatic insults, such as inflammation (accompanied by the release of proinflammatory cytokines), oxidative stress (associated with increased levels of reactive oxygen species) and endoplasmic reticulum stress, which in turn propagate inflammatory and fibrogenic responses within the liver¹³. This initially simple model has evolved considerably in the past decade as a result of the recognition that factors originating in other tissues, such as adipokines, immune cells and metabolites produced by gut microbiota, make important contributions to the pathogenesis of NASH. These same factors are also implicated in the progression of NAFLD towards more severe stages^{14,15} and act in concert with genetic markers of susceptibility to NAFLD¹⁶.

The global prevalence of NAFLD has now reached epidemic proportions; this condition is estimated to affect 15–31% of the total population when detected by indirect (non-histological) methods. The highest rates of NAFLD are reported in South America (30%) and the Middle East (32%), followed by Asia (27%), the USA (24%), Europe (23%) and Africa (14%) with ethnic disparities¹⁷. North Americans of Hispanic descent are more prone to NALFD (2 to 3-fold) when compared to the overall population. Moreover, ~20% of individuals with NAFLD probably also have features of NASH¹⁸. Although NASH is also detected in lean patients, in whom the condition shows a strong ethnic bias to vary between 10-15% in Asia, 15% in Europe and 37% in USA. While still elusive, reasons for these differences probably reflect genetic, social and dietary influences^{19,20}. The prevalence of NASH is 2 to 3 fold higher in obese individuals than in lean ones²¹ and in patients with type 2 diabetes mellitus (T2DM) than in the background population^{22,23}. These two groups accordingly account for a high proportion of the patients with advanced fibrosis. Patients with fibrosis are more likely to develop liver-related adverse outcomes such as hepatocellular carcinoma (HCC) as well as liver-unrelated comorbidities such as cardiovascular disease (CVD)^{24,25}. Thus, NASH accounts for an ever-increasing burden on health-care systems.

A wealth of studies of the various dysfunctional organs and cell types that contribute to progression of NAFLD has led to the identification of multiple signalling and metabolic pathways that

promote liver dysfunction and fibrosis, among which insulin resistance is a major initiating pathological event that drives de novo lipogenesis in the liver²⁶⁻²⁸. Despite tremendous efforts to develop drugs that interfere with these dysregulated metabolic, inflammatory and other biological processes, so far these therapeutic approaches have not been successful in either preventing or halting the progression toward NASH and fibrosis²⁹. This failure is probably a reflection of the multifactorial and heterogeneous nature of this disease, as well as the lack of tools for its accurate diagnosis and for quantitative disease staging^{30,31}. In addition, sex is rarely considered as a biological variable in these numerous and interesting studies, despite the reported sexual dimorphism of the gut microbiota³², the innate immune system³³ and the liver itself. As a result, in-depth studies conducted in relevant models are necessary to fully understand the natural history of NAFLD, and to identify sex-dependent determinants of NAFLD progression.

This Review highlights the evidence from preclinical and clinical studies indicating that sex is an important biological variable in hepatic physiology and NAFLD. This aspect becomes highly relevant as studies' accuracy reaches the single cell level, providing unique opportunities to provide a comprehensive atlas of cellular modifications in NAFLD.

[H1] Sexual dimorphism in the liver

Although some essential hepatic functions (gluconeogenesis, bilirubin detoxification, iron storage and production of clotting factors) are equally carried out by the liver in male and female individuals, others clearly differ according to sex. Indeed, sex-specific differences in hepatic function have long been observed in sexually mature oviparous animals and mammals³⁴. Sex-specific production of hepatic proteins such as vitellogenin, α 2-microglobulin, bile acids, xenobiotic transporters³⁵ and cytochrome P450 (CYP) enzymes involved in metabolism of sex steroids³⁴ has been described in various animal models and in humans. In most cases, sex-specific differences in levels of protein production are reflected in gene expression levels, and sex-biased expression of about 1,000 mRNA transcripts has been described in liver cells from male versus female individuals³⁶. These features, which are partly a result of the exquisitely close coupling of metabolism to reproductive needs (a necessity for successful pregnancy in female individuals), orientate the female liver towards a highly efficient metabolic phenotype³⁷ characterized by increased mitochondrial biogenesis³⁸.

In mice, hepatic sexual dimorphism is also observed at the metabolomic level³⁹ and this functional adaptation has been ascribed to the transcriptional control of gene expression by the pulsatile (in males) or constant (in females) secretion of growth hormone (GH)⁴⁰. The GH-STAT5b pathway regulates CYP enzymes involved in metabolism of xenobiotic molecules and sex steroids⁴¹ in a sex-specific and species-specific manner, such that sexual dimorphism is highly pronounced in rats

and attenuated but still substantial in humans^{42,43}. This pathway is an important contributor to liver sexual dimorphism, in addition to the androgen and oestrogen signaling pathways^{44,45}.

Over the years, additional regulatory mechanisms have been implicated in the control of sex-specific liver functions, including the circadian clock^{46,47}, the bile acid-activated farnesoid X receptor (FXR) pathway⁴⁸⁻⁵⁰ and the gut microbiome, which exacerbates sexual dimorphism in the mouse liver by controlling the secretion of GH and sex hormones^{39,48,51}.

[H1] Molecular basis of liver sexual dimorphism

All the pathways that control sex-specific liver functions are deeply interconnected^{39,52} but how they cooperate to establish molecular sexual dimorphism in the liver remains to be fully characterized. The mechanism through which GH determines hepatic sexual dimorphism is under intense investigation⁵³. For instance, the level of expression of genes that regulate lipid, cholesterol and drug metabolism in the adult liver is determined by the GH secretion pattern⁴³, which in rats is established by androgen exposure (which causes persistent epigenetic changes) during the neonatal period^{54,55}. Importantly, gut microbiota-derived metabolites are also required to establish the male-specific, pulsatile GH secretion pattern in mice, and germ-free mice display a less pronounced transcriptional sexual dimorphism³⁹. In female rats, by contrast, the absence of neonatal androgen exposure means that GH secretion by the pituitary gland is constant, which leads to a desensitized, low activity of the JAK2–STAT5b pathway. Of note, STAT5a participates in GH-mediated signaling to a lesser extent than does STAT5b, as STAT5a is expressed only in minute amounts in the liver⁵⁶. Accordingly, transient phosphorylation and activation of STAT5b is considered to be a masculinization event that drives upregulation of male-specific genes as well as downregulation of female-specific genes⁵⁷ (Figure 1). GH dictates chromatin opening in a sex-dependent manner, which is accompanied by deposition of specific histone marks at gene-regulatory regions without altering the pericentral-to periportal zonal expression pattern of sex-specific genes⁵⁸. Therefore, metabolic features of the liver are specified by brain hormones.

Sex hormones (notably 17 β -oestradiol, referred to herein as oestradiol) also play an important part in the regulation of metabolic genes with sex-biased expression. The results of hormone replacement therapy studies in humans as well as gene deletion experiments in mice concur that oestrogens suppress hepatic de novo lipogenesis, increase fatty acid oxidation, increase production of VLDL triglycerides, decrease the synthesis of cholesterol (via increased clearance of plasma apolipoprotein B particles) and increase the synthesis of cholesterol-derived bile acids^{59,60}. Thus, oestradiol has a protective effect in female livers that is also observed [in male livers as a result of aromatase-mediated conversion of testosterone to oestradiol, which safeguards hepatic insulin sensitivity, modifies bile acid synthesis and limits the bile acid pool size⁶¹⁻⁶⁴.

Gene deletion experiments and other studies in mice are mostly supportive of a contribution of nuclear estrogen receptor α (ESR1) and to a lesser extent a contribution of G-protein coupled oestrogen receptor (GPER) to the protective effects of oestradiol, but neither ESR2 (ER β) nor nuclear androgen receptor (AR) contribute to this mechanism⁶⁵⁻⁶⁷. The role of androgens and AR in regulation of hepatic lipid metabolism (and hence their contribution to steatosis) has been studied because of the highly conserved pro-steatotic effect of orchidectomy in many species⁶⁵. Both testosterone and dihydroxytestosterone can act as anti-steatotic agents, but whether aromatization to oestradiol plays a part in this effect is unclear. The findings of gene deletion studies in mice are inconsistent but generally point to a protective role of AR in males and no effect of AR in females⁶⁵. Of note, other transcription factors, such as aryl hydrocarbon receptor (AHR), pregnane X receptor (PXR/NR1I2) and farnesoid X receptor (FXR/NR1H4) also participate in the establishment of liver sexual dimorphism by controlling male sexual maturation, a process under the control of gut microbiota-produced metabolites that regulate AHR, PXR and FXR transcriptional activities³⁹. Importantly, the regulation of genes involved in metabolism is partly subject to circadian oscillation, which is itself controlled by sex. A few studies have found increased resilience of female individuals to circadian rhythm disruption⁶⁸, which is a predisposing factor for cardiometabolic diseases including NAFLD^{69,70}.

[H1] Sexual dimorphism in NAFLD and NASH

NAFLD, which in its most rapidly progressive form leads to NASH and fibrosis, is associated with an increased risk of liver-related and cardiovascular-related comorbidities in adults^{71,72} (Figure 2). The early manifestations of NAFLD (that is, NAFL or steatosis), have a higher prevalence in men than in women irrespective of age^{73,74}. In healthy men, the risk of NAFLD is inversely correlated with blood levels of oestradiol⁷⁵. However, the prevalence of NAFLD is also steadily increasing in adolescents⁷⁶ and both the development of NAFLD and its progression to fibrosis show dose-dependent correlations with fructose intake⁷⁷. Paediatric NAFLD is histologically characterized by portal inflammation that can progress to fibrosis in the absence of detectable hepatocyte degeneration or ballooning, in contrast to NAFLD in adults, which is characterized by mostly lobular inflammation and considerable ballooning⁷⁸. Interestingly, levels of SHBG are inversely correlated with NAFL severity and although oestradiol levels do not correlate with NAFLD stage, testosterone levels are inversely correlated with fibrosis severity in boys⁷⁹. Therefore, sex hormones are likely to also exert major effects in the paediatric population, but will not be further discussed in this Review owing to the scarcity of epidemiological and mechanistic studies⁸⁰.

In general, men have more advanced grades of NASH than women^{81,82}, and men are also more prone than women to develop fibrosis⁸³. NASH-related HCC is diagnosed 2–4 times more often in men than women, and this sex bias is also observed in mouse studies, which suggests that the

increased susceptibility to HCC observed in men is unlikely to be due to increased exposure to risk factor(s)⁸⁴. This protection from the development of HCC is similarly unlikely to reflect a globally higher resistance of the female liver to injury, as some other liver pathologies, such as alcoholic liver disease and primary biliary cirrhosis, develop more aggressively in women than in men⁸⁵. As with the physiological aspects of liver sexual dimorphism, little is known of the intrinsic molecular mechanisms underlying the exacerbated development of NASH in males. Although genetic studies suggest that susceptibility to NASH and fibrosis (assessed by non-invasive methods) are heritable traits^{16,86}, known risk alleles in the coding or noncoding regions of *PNPLA3*, *TM6SF2*, *GCKR*, *MBOAT7*, *SLC39A8*, *SLC30A10* and *HSD17B13* genes are not associated preferentially with either sex^{87,88}. As the metabolic features of genetically driven NASH are distinct from those of metabolically driven NASH^{89,90}, the genetic type will not be considered further in this Review.

Sex hormones (notably oestrogens) have a fundamental role in liver protection, as the prevalence of NASH in postmenopausal women (average age 51 years) in the USA is equivalent to that in men⁹¹, and premature menopause increases the risk of both NASH and fibrosis⁹². However, studies of the effects of hormone replacement therapy in humans have not yet formally established exogenous estrogens as liver-protective drugs, despite evidence of their positive effects in rodents⁹³. Conversely, the results of a retrospective study suggest that the excess androgens present in women with polycystic ovary syndrome render them more likely to develop NAFLD⁹⁴. Clinical trials of anti-androgen treatment in patients with HCC have failed to show any benefit, but were mostly carried out in patients with late-stage, virally induced tumours⁹⁵.

[H1] Molecular explanations

As described in a number of recent comprehensive reviews, the natural history of NAFLD involves slow progression (over decades) from steatosis to steatohepatitis and ultimately to fibrosis, albeit with considerable inter-individual variation and spontaneous reversibility^{18,72,96-99}. Progression through each stage increases the probability of worsening liver function, with advanced fibrosis being the most powerful predictor of liver-related or overall mortality^{25,100-102}. Each stage is characterized by specific hepatic histological features¹⁸ and is associated with prominent cellular (dys)functions that can be identified either by transcriptomic profiling of human bulk liver tissue or at the single-cell level¹⁰³⁻¹⁰⁹. Both clinical and experimental studies suggest a causative link between NASH and fibrogenesis^{26,110}.

From this wealth of data consensus has emerged: that steatosis is linked to dysregulated expression of genes related to lipid storage, lipid and amino acid metabolism; that steatohepatitis is linked to dysregulation of genes involved in inflammation; and that fibrosis is linked to dysregulation of genes involved in extracellular matrix remodeling (Figure 2). Owing to considerable variations in

sample size, cohort constitution and technical factors, the results of these studies show little or no agreement as to whether the identified genes have a causative role in human disease. A core set of genes that are dysregulated in NASH or fibrosis has been identified through meta-analyses of mouse and human transcriptomic data, and the genes included in this core set mostly have functions involving the control of protein stability, inflammation or extracellular matrix architecture^{106,109,111}. A random forest-based approach has identified male-specific and female-specific NASH gene signatures that had fairly high predictive capacity in independent cohorts¹¹². However, these analyses could not establish whether physiologically sex-biased gene expression¹¹³ is perturbed in individuals with NASH, as the initial cohort included morbidly obese patients but not lean controls.

Metabolomic approaches have also identified NAFLD-induced alterations in blood levels of lipids and amino acid metabolites. The results of these studies are in general agreement that blood levels of lipid species increase during the progression of NAFLD^{114,115}. By contrast, some but not all studies reported altered levels of bile acids during progression of NAFLD, and this lack of consensus probably related to the use or not of cohort stratification criteria based on insulin resistance has led to controversial interpretations regarding the implication of bile acids in NAFLD pathogenic events. Interestingly, a multi-omics study of non-diabetic women reported, like others¹¹⁶⁻¹¹⁸, increased levels of branched chain amino acids in individuals with NAFLD. Synthesis of branched chain amino acids is catalyzed by the gut microbiota, which (in patients with steatosis) has low microbial gene richness and increased genetic potential for dietary lipid processing and endotoxin biosynthesis. In gut microbiota transplant experiments conducted in male mice, transfer of such low-richness gut microbiota triggered anaplerosis of the tricarboxylic acid cycle and ultimately steatosis¹¹⁹. In an independent study, increased levels of branched chain amino acids were specific to women, whereas men exhibited decreased levels¹²⁰. This observation might provide a basis for the relative resistance of women to NAFLD, as in some studies supplementation with branched chain amino acids has been shown to be protective in preclinical models of NASH and also in patients with NASH. However, whether branched chain amino acids are beneficial or detrimental for liver health is still debated¹²¹.

Finally, HCC also occurs more frequently in men than in women¹²². HCC is characterized by a sex-biased transcriptional pattern¹²³ to which GH, sex hormones, their cognate nuclear receptors and associated pioneer transcription factors contribute¹²². The incidence of HCC is increased in patients with advanced NASH or cirrhosis¹²⁴, but whether NAFLD-related HCC also exhibits sexual dimorphism is not clearly established^{125,126}. In one rodent study, abrogated expression of the liver-specific P1 isoform of HNF4 α , which has an important role in the control of lipid, glucose and drug metabolism in the liver^{127,128}, suggested that HNF4 α is critically involved in establishing the sex-biased development of NASH-induced HCC¹²⁹. Whether the proposed mechanism, which involves IL-6 and STAT3, is also operative in humans is unknown.

[H1] Clinical considerations

Ample evidence supports the concept that sex is an important biological variable influencing the progression of NAFLD. However, the effect of sex continues to be overlooked even in the most recent publications relating to preclinical experiments, biomarker discovery and validation studies and even, surprisingly, in clinical trials in this field. This oversight is evident not only in the adult population, but also in paediatric and lean populations with NASH, which are much less thoroughly studied than the obese adult population. Although the need for improved stratification of patients with NASH is acknowledged both in clinical practice and in drug development, such stratification is rarely implemented in daily practice; therefore, insights might be gained from de-segregation of human transcriptomic data by sex¹¹². Drug-based treatments options for NASH do not yet exist but the possibility of sex-based dosage optimization will need to be considered in the future. Early detection of individuals predisposed to develop NASH integrates parameters such as insulin resistance, but these parameters are also strongly influenced by sex^{130,131} and interestingly, by GH, which is generally considered an antagonist of the actions of insulin. In addition to being sex-dependent, the secretion of GH has a circadian cycle that is affected by altered gut microbiota and sleep disorders, both of which contribute to the development of metabolic syndrome¹³².

[H1] Research needs and future perspectives

Complex multi-organ dysfunction contributes to the development of liver pathology in patients with NAFLD. Each of the affected components (liver, intestine, the gut microbiome, adipose tissue, immune cells and skeletal muscle) also has sex (hormone)-dependent physiology. Although most mechanistic data have been obtained from studies in male rodents, the sexual dimorphism of white adipose tissue depots is obvious from both histological and molecular viewpoints^{133,134}. Crosstalk between the liver and white adipose tissue, exemplified by the occurrence of white adipose tissue inflammation preceding the development of NAFLD, has not yet been considered a sex-dependent process¹³⁵. However, in mice, regulatory T cells (which regulate immune responses in visceral adipose tissue and exert tempering effects in NASH¹³⁶) differ phenotypically and molecularly between the sexes¹³⁷. In addition, resident and infiltrating macrophages in white adipose tissue and in the liver have important roles in NASH¹³⁸ and sex hormones strongly influence the phenotypes of these cells³³.

Apart from gonadal sex, chromosomal sex (through X-escape genes — genes on the inactivated X chromosome that are nonetheless transcribed to produce RNA levels $\geq 10\%$ of the expression of their counterparts on the active X chromosome) could also influence organ pathophysiology in NAFLD. The ability of white adipose tissue to expand, and specifically the ability of adipocytes to proliferate and differentiate in a metabolically challenging environment, also affects glucose homeostasis. Histone demethylase KDM5C, which is encoded on the X chromosome and

escapes X-chromosome inactivation, controls the proliferation of adipocytes¹³⁹ and contributes to the sex-specific expandability of white adipose tissue¹⁴⁰. This circumstance raises the interesting possibility that X-linked gene expression levels might affect insulin resistance and hence progression of NAFLD, at least in its early stages. However, this hypothesis remains to be formally tested. Another parameter that is rarely taken into account in preclinical studies is ageing, mostly owing to cost and/or logistical constraints. Indeed, most preclinical studies of diet-induced NAFLD or NASH are conducted in young (usually male) animals and the disease barely reaches the fibrotic stage.

Finally, most knowledge of human NASH has been collected through transcriptomic data. Other causes of liver dysfunction, such as abnormal activation or inactivation of signalling pathways, might be overlooked. As an example, the GH–JAK2–STAT5 pathway strongly affects liver responses to metabolic and chemical insults in rodents¹⁴¹, yet the contributions of this and other signalling pathways to human NAFLD have not been directly tested using powerful approaches such as phosphoproteomics¹⁴². These knowledge gaps undoubtedly call for improved characterization of the animal models used in NASH research, the use of complementary techniques (such as integrative omics studies^{143,144}) and for careful translation of observations made therein to human pathology¹⁴⁵.

[H1] Conclusions

The liver is a sexually dimorphic organ and the pathological events leading to NAFLD progression in the two sexes are likely to differ, at least in part. This situation warrants a thorough investigation of sex-specific molecular mechanisms. In preclinical studies, the selection of appropriate experimental options (such as use of male or female animals) needs to go beyond the expectation of maximal and stable responses. Therefore, a full understanding of NAFLD progression and the identification of optimal therapeutic strategies requires the inclusion of sex as a critical parameter in driving pathological events.

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Author contributions

The authors contributed to all aspects of the article.

Competing interests

The authors declare no competing interests.

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Supplementary information

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Key points

- **Liver pathophysiology is sexually dimorphic**
- **Male individuals predominantly show more severe stages of NAFL, NASH and fibrosis**
- **Individual variations in humans require large cohorts to identify sex-specific features**
- **(Pre)clinical investigations of NAFLD and of fibrosis rarely consider sex as a biological variable**
- **The time (and technologies) are ripe for investigating the sexual dimorphism of liver diseases in time and space**

Figure 1: Main physiological pathways involved in liver sexual dimorphism. The growth hormone (GH) and sex hormone signaling pathways are shown, as well as additional feedback loops controlling the release of GH from the pituitary gland. The principal biological outputs are also indicated.. GHRH, growth hormone-releasing hormone; HPA, hypothalamic–pituitary–adrenal axis; IR, insulin receptor; GHR, growth hormone receptor; NEFAs, non-esterified fatty acids; E2, 17 β -oestradiol; *CYP19A1*, the gene encoding aromatase; JAK2, Janus kinase 2; STAT5, signal transducer and activator of transcription 5; ER, estrogen receptor (encoded by *ESR1*); IGF-I, insulin-like growth factor 1; *PPARG*, the gene encoding peroxisome proliferator activated receptor- γ ; SREBP1c, sterol regulatory element-binding protein 1c (encoded by *SREBF1*).

Figure 2: Progression of NAFLD is influenced by sexual dimorphism. Various organ responses and dysfunctions influence the progression of nonalcoholic fatty liver disease (NAFLD). Only sexually dimorphic features that are established or at least partially described in the literature are indicated. NEFAs, non-esterified fatty acids; NAFL, non-alcoholic fatty liver; ECM, extracellular matrix; HCC: hepatocellular carcinoma.

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