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Review

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Mini liver organoid models emulating metabolic dysfunction-associated steatotic disease development: gradually emerging beneath the iceberg

Ting Feng[1,#], Jiaming Li[2,#], Xiaoyi Ma[3], Junzhao Ye[4]

[1]Department of Gastroenterology, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou 510080, Guangdong, China.
[2]Department of Gastroenterology, The Fifth People's Hospital of Shunde District, Foshan 528000, Guangdong, China.
[3]Department of Geriatrics, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong, China.
[4]Department of Gastroenterology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, Guangdong, China.
[#]Authors contributed equally.

Correspondence to: Prof. Xiaoyi Ma, Department of Geriatrics, The First Affiliated Hospital, Sun Yat-Sen University, 58 Zhongshan 2nd Road, Yuexiu District, Guangzhou 510080, Guangdong, China. E-mail: maxy85@mail.sysu.edu.cn; Prof. Junzhao Ye, Department of Gastroenterology, The First Affiliated Hospital, Sun Yat-Sen University, 58 Zhongshan 2nd Road, Yuexiu District, Guangzhou 510080, Guangdong, China. E-mail: yejzh@mail2.sysu.edu.cn

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Abstract

Tissue spatial interaction in the liver, including the interplay among immune cells, blood vessels, sinusoidal endothelial cells, bile ducts, stellate cells, hepatic progenitor cells, and hepatocytes, is crucial not only for normal organogenesis but also for disease development. Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as the most prevalent liver disease, with heterogeneous mechanisms involving cross-talk between hepatocytes and stromal cells under metabolic stress, inflammation, and fibrosis. Developing 3D coculture liver organoid platforms provides novel insights into the complex links of cell communication during disease progression. Thus, we recapitulate liver organoids as models for indicating reconstructed inter-tissue interactions of MASLD, highlighting the potential of transplanting liver organoids as a therapeutic application.

Keywords: MASLD, liver organoid, 3D coculture, disease progression

INTRODUCTION

Metabolic dysfunction associated steatotic liver disease (MASLD), originally termed non-alcoholic fatty liver disease (NAFLD), is closely associated with the metabolic syndrome and associated extrahepatic complications, and exhibits metabolic reprogramming-related liver injuries in multiple complexed interactions among epigenetic and epitranscriptomic modifications[1,2]. MASLD represents a severe global health problem, with its high prevalence and continuous progressions as key drivers of end-stage liver disease and cardiometabolic disease[3,4]. This progression is characterized by a complex interplay of insulin resistance, lipotoxicity, and low-grade systemic inflammation, and is consistent with the severity of MASLD histology. MASLD has also been shown to be a leading cause of liver transplantations[5,6]. Liver transplantation remains the unique cure for end-stage liver disease in MASLD, but the supply falls short of the unmet demand[7]. Therefore, artificial liver tissue with biomedical function has become a promising alternative to whole liver transplantation. Over the past decades, extensive research has focused on the mechanisms of liver regeneration, leading to the development of stem cell therapy-derived organoids and related emerging technologies[8,9]. Advances in these models have, in turn, greatly deepened our understanding of the dynamic interactions among different cell types and their metabolic changes in chronic liver diseases[10].

The liver, as the most important metabolic organ, consists of a heterogeneous structure that supports various physiological functions, which encompasses liver lobules and parenchyma of hepatocytes regenerated by liver progenitor cells (LPCs), the bile drainage system of cholangiocytes, blood vessels, essential matrix components, and both resident and circulating immune cells[11]. Traditional mouse models might not mimic key aspects of patient liver metabolism due to distinct dietary habits and genetic backgrounds[12]. These include discrepancies in liver size, metabolic rate, and lipid metabolism pathways[13]. For instance, mice have a faster metabolic rate and a different lipid accumulation pattern in the liver compared to humans. Additionally, the regenerative capacity of the liver in mice is more robust, which can affect the progression and outcomes of MASLD in ways that are not reflective of the human liver with steatosis. These differences can limit the translatability of findings from mouse models to human liver disease, particularly in terms of understanding disease progression and therapeutic responses. Additionally, the high costs associated with culture time and resources limit the application of primate models. Cell lines are flawed because they lose the integrity of their microenvironment, particularly due to the absence of complex structures or signals from other cells, as well as altered metabolic adaptations during immortalization[14]. At present, one potential solution to the shortage of donor liver is xenotransplantation, including developments in gene-edited pigs and cold storage of organs; however, recipient immune responses and infection related to immune system inhibition remain challenges in translating xenotransplantation into clinical practice[15].

Thus, liver organoids derived from humans would more accurately simulate pathological conditions compared to traditional models by capturing the progression of stem cell self-organization into three-dimensional (3D) tissue structures through cell-cell and cell-matrix interactions, especially from a metabolic perspective[16]. The rapid advancement of liver organoids has helped elucidate the complicated metabolic activities of nutrients, drug chemical modifications, oxidative stress, and bile secretion in MASLD, compared to a normal liver[17]. More importantly, liver organoids have become irreplaceable in decoding the role of immune reaction cells in MASLD, which previously posed major obstacles in research due to traditional two-dimensional (2D) coculture systems.

In this review, we comprehensively report the current application of biologically engineered liver organoid models and how they facilitated the understanding of the mechanisms of MASLD, the development of novel

therapies, and the potential for achieving personalized medicine.

DIFFERENT GENERATION STRATEGIES FOR ESTABLISHING LIVER ORGANOIDS

Liver organoids are defined as 3D structures mimicking the native liver bud architecture and function *in vitro*. They originate from the colonization of stem cells, progenitor cells, and/or differentiated cells, which self-organize through cell-cell and cell-matrix interactions, recapitulating aspects of liver organoids[18]. They possessed the ability to self-proliferate, renewing their composition and expanding. Additionally, they exhibit a stable degree of maturation and perform part functions, including albumin/coagulation factor synthesis, midazolam metabolism, ammonium elimination, low density lipoprotein uptake, and glycogen accumulation. The current classification system for liver organoids and their nomenclature is based on the originating cell types, such as Leucine-rich repeat-containing G protein-coupled receptor 5 positive (Lgr5+) stem cells, primary hepatocytes (PHs), patient-derived xenografts, or the coculture of cells derived from at least two germ layers[19].

Hepatocyte organoids [Figure 1]

Lgr5+ stem cells

The Wnt signaling pathway targets the expression of Lgr5+, a well-characterized stem cell marker in embryos, and a representative clonal marker, which could initiate the expansion of a subpopulation of LPCs when the liver experiences injury, but it is not active in homeostatic status[20,21]. After isolating single cells from liver samples, the cells were mixed directly with Matrigel for culture in a specific organoid culture medium containing advanced DMEM/F12 (Invitrogen), supplemented with 1% N2 (Invitrogen), 2% of B27, 1.25 μM N-acetylcysteine, 10 nM gastrin (Sigma-Aldrich), 50 ng/mL epidermal growth factor (EGF), 10% R-spondin-1 (RSPO1, as Wnt/β-catenin signaling pathway activator), 100 ng/mL fibroblast growth factor 10 (FGF10), 10 mM nicotinamide, and 50 ng/mL hepatocyte growth factor (HGF)[22,23]. During the first 8-12 days of culture, organoids were supplemented with 10 μM Y-27632 (Rho kinase-inhibitor), Noggin, and Wnt3a-conditioned medium[24]. After 2 days of culture, a supplement of bone morphogenetic protein 7 (BMP7) and culture in spinner flasks might greatly improve growth efficiency by enhancing oxygenation[25]. Importantly, inhibition of Notch and transforming growth factor beta (TGF-β) signaling might direct the differentiation of hepatocytes into biliary cells.

Primary liver cells

A 3D culture based on a collagen matrix could overcome the limitations of traditional 2D models, which often result in rapid epithelial-mesenchymal transition and dedifferentiation, leading to the loss of liver-specific functions. A dome-shaped collagen matrix would further assist in liver function preservation[26]. PHs were isolated from donor pigs through *in situ* collagenase perfusion[27]. When cocultured with human umbilical vein endothelial cells (HUVECs) overexpressing RSPO1, these dispersed liver cells self-assemble into organoids within 24 h in a Rocker culture system (a rotation bioreactor continuously rocked at 10 rpm). Coculture with HUVECs that mimic liver sinusoidal endothelial cells (LSECs) helps replicate the natural liver microenvironment by providing necessary exogenous signals that support hepatocyte function and longevity.

Another strategy for constructing rapid self-assembly mini-livers (RSALs) involved designing a 3D scaffold using fibrinogen and thrombin, both of which physiologically served as matrices that facilitate platelet aggregation during the coagulation process[28]. PHs dispersed in a fibrinogen solution (> 1 mg/mL) were added to the thrombin solution (over 1 U/mL) on an agarose-coated plate. Remarkably, neovascularization was observed in the large RSALs (with diameters reaching 5.5 mm) when they were transplanted to the mesentery of a mouse model.

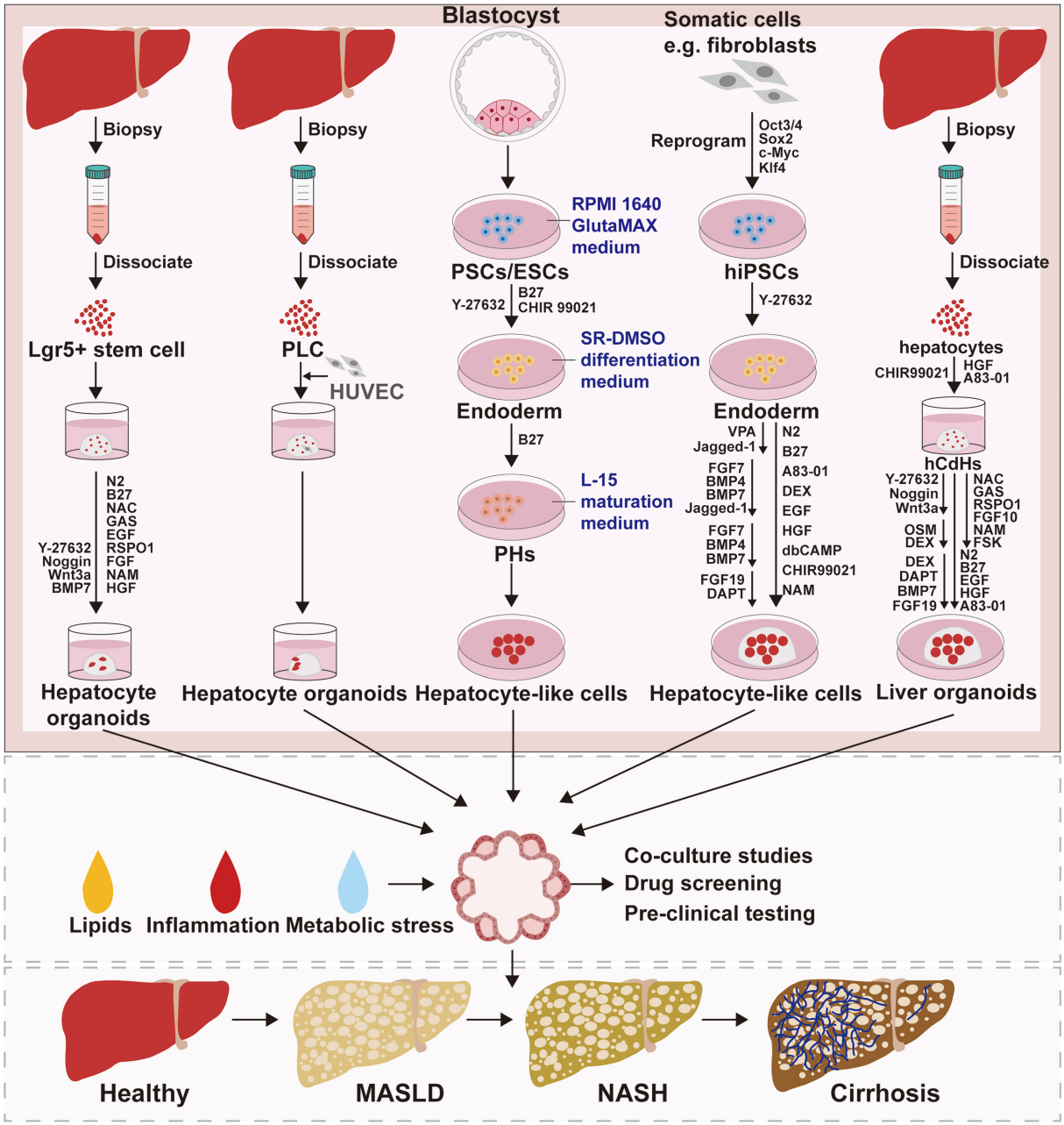

Figure 1. Hepatocyte organoids originated from Lgr5+ stem cells, PLC, human pluripotent stem cells, human induced pluripotent stem cells, and human chemically-derived hepatic progenitors for investigating MASLD progression. MASLD: Metabolic dysfunction-associated steatotic liver disease; NASH: non-alcoholic steatohepatitis; PSCs: pluripotent stem cells; ESCs: embryonic stem cells; hiPSC: human-induced pluripotent stem cells; PLC: primary liver cells; HUVECs: human umbilical vein endothelial cells.

Human pluripotent stem cells

The differentiation protocol for directing human pluripotent stem cells (PSCs) toward hepatocyte-like cells and organoids follows a four-stage process over 17-20 days[29]. Human plateable hepatocytes (PHs) are isolated using Accutase and plated onto Geltrex-coated tissue culture plates. On Day 0, PSCs are cultured in RPMI 1640 GlutaMAX medium supplemented with Y-27632. By Day 1, the medium is enriched with B27 supplement and CHIR 99021 (a selective GSK-3α/β inhibitor that activates the Wnt/β-catenin pathway), which induces a typical definitive endoderm morphology, with the expression of protein markers like CER1,

GATA4, SOX17, and HHEX. From Day 2 to Day 7, hepatoblast-specific differentiation is induced using only the B27 supplement and SR-DMSO differentiation medium, as evidenced by markers such as PROX1, CEBPA, TTR, and AFP. From Day 7 to Day 20, the cells are cultured in L-15 maturation medium to promote hepatocyte maturation, resulting in hepatocyte-like morphology and the expression of fetal hepatic marker AFP and mature hepatic markers such as ASGR1, CYP3A4, A1AT, ALB, APOA2, TDO, and TTR. Throughout the entire process, a suspension culture method is employed (orbital shaking at 70 rpm in a 37 °C, 5% CO_2 incubator), which facilitates the formation of primitive streak/mesendoderm organoids from PSC aggregates. Single-cell transcriptome analysis of Day 7 organoids reveals a subset of MESP1-positive cells, suggesting the presence of mesodermal STM31, which later gives rise to hepatic stellate cells (HSCs).

Human-induced PSCs

Human-induced PSCs (hiPSCs) are generated by reprogramming somatic cells through the delivery of reprogramming factors, including the translational factors Oct3/4, Sox2, c-Myc, and Klf4, which are either permanently integrated into the host cell genome or continuously activated by small molecules. Due to their potential to generate an unlimited supply of human embryonic-like cells without the need for stem tissues, hiPSCs have become one of the most widely reported methods for establishing liver organoid models[30]. Commercial Reagent kits for inducing liver organoid differentiation have been developed[31]. On Days 0-3, iPSCs were treated with the STEMdiff Definitive Endoderm Kit (Stem Cell Technologies) and 50 μM Y-27632. On Day 4, the cells were treated with a culture medium containing Glutamax supplement, B27, 20 ng/mL BMP4, and 10 ng/mL FGF2 in Advanced DMEM/F12 (Kit 2) for 6 days. Following this, the medium changed to Advanced DMEM/F12 supplemented with N2 supplement, B27, 50 nM A83-01, 30 μM dexamethasone, 5 μM CHIR99021, 500 nM valproic acid, 50 ng/mL EGF, 20 ng/mL HGF, 40 ng/mL Jagged-1, 300 ng/mL dbCAMP, and 10 μM nicotinamide in Matrigel for 8 days. A shaking culture system was used with a modified medium formula (Kit 3), which excluded valproic acid and incorporated FGF7 (25 ng/mL), BMP4 (50 ng/mL), and BMP7 (20 ng/mL) for another 8 days. Then, the cultural medium was replaced with Kit 3, adjusting the concentration to 2 μM CHIR99021 and 20 ng/mL BMP4, and removing Jagged-1 for 12 days (Kit 4). For the following 8 days or longer, components of Kit 4, including BMP4, BMP7, and FGF7, were replaced with FGF19 (25 ng/mL) and DAPT (5 μM). Inhibiting the DIO2-T3 signaling pathway might shift the expression of key transcription factors, such as PROX 1 and HHEX, reducing the proportion of hepatocyte-like cells while increasing cholangiocyte-like cell amounts within the liver organoid. hiPSC-based liver organoids have been enhanced with the overexpression of UDP-glucuronosyltransferase family 1 member A1 through Lipofectamine 3,000 transfection[32]. Another method to enhance the functionality of these organoids might involve the use of decellularized liver scaffolds, which are created by disassociating cells from whole liver tissues, leaving behind the extracellular matrix[33]. Furthermore, adding platelet-derived growth factors (PDGFs) on Day 10 may promote hepatic spheroid formation by activating PDGF receptors and stimulating the expression of NECTINs and NECL5, which are involved in adherens junctions[34]. Interestingly, the use of micropatterned cell-adhesion substrates, such as polydimethylsiloxane (PDMS) film-covered polyethylene glycol surfaces, as a cultural material may enable the generation of high-throughput, homogenous liver organoid models[35].

Human chemically-derived hepatic progenitors

Human chemically-derived hepatic progenitors (hCdHs) were derived from single hepatocytes from the liver of healthy donors and reprogrammed into bipotent progenitor cells using two small molecules, A83-01 and CHIR99021, along with HGF[36]. The hCdHs were mixed with Matrigel and incubated in an organoid expansion medium, which was adjusted from Advanced DMEM/F12 media and supplemented with 1 % N2, 1 % B27, N-Acetylcysteine (1.25 mM), gastrin (10 nM), EGF (50 ng/mL), 10 % RSPO1 conditioned media, FGF10 (100 ng/mL), HGF (25 ng/mL), nicotinamide (10 mM), A83-01 (5 μM), and FSK (10 μM) with

Noggin (25 ng/mL), Wnt3a (100 ng/mL), and Y-27632 (10 μM) added. After 72 h, Noggin, Wnt3a, and Y-27632 were removed from the culture media. Collagen-coated plates were then used for 3D culture, and organoid expansion was continued for 6 days in medium containing Oncostatin M (20 ng/mL) and dexamethasone (10 μM). Next, liver organoids generated from hCdHs (hCdHOs) were cultured in organoid expansion medium supplemented with BMP7 (25 ng/mL) for 5 days, followed by a change to differentiation media consisting of Advanced DMEM/F12 media supplemented with B27 (2%), N2 (1%), EGF (50 ng/mL), HGF (25 ng/mL), A83-01 (0.5 μM), DAPT (10 μM), dexamethasone (3 μM), BMP7 (25 ng/mL), and FGF19 (100 ng/mL) for 15 days. Single-cell transcriptomics analysis indicated that hCdHs regained phenotypic features of hepatocytes and cholangiocytes, as characterized by the expression of EpCAM, LGR5, SOX9, KRT7, and KRT19[37].

These hepatocyte organoids, when stimulated with lipids, inflammation, and metabolic stress, can be used for coculture studies, drug screening, and preclinical testing, aiming to investigate the mechanisms of MASLD progression to non-alcoholic steatohepatitis (NASH) and liver cirrhosis.

Multi-lineage liver organoids [Figure 2]

The aggressive disease behavior in MASLD highlights the cell–niche interactions that are the basis for the key events occurring *in vivo*[38]. Developing whole human liver tissues is expensive and time-consuming, making them unsuitable as experimental modalities. These obstacles could be addressed by using different patient-derived cells to establish primary structures that essentially recapitulate the function and molecular pattern of the tissue.

hiPSC-derived 3D hepatic organoids-macrophage model

Human iPSCs (HDF01) were chosen as the source for inducing pre-macrophage differentiation[39]. They were initially cultured with mTeSR1™ (Stem Cell Technologies, Vancouver, Canada) on Matrigel®-coated dishes (Corning, Tewksbury, MA, USA). The culture environment was then replaced with a medium supplemented with BMP4 (80 ng/mL) for 4 days. Serial hematopoietic cytokines, including M-SCF (100 ng/mL), GM-CSF (25 ng/mL), and Flt3L (50 ng/mL), were added for 11 days, followed by a maturation phase in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and M-CSF (100 ng/mL) for 7 days. HiPSC-derived hepatic organoids were dissociated into single cells using TrypLE and mixed with pre-macrophages. The resulting mixture was then cocultured in a medium composed of advanced DMEM/F12, N2, B27, 3 μM CHIR99021, 10 ng/mL BMP4, 10 ng/mL FGF7, 10 ng ml/L FGF10, and 50 nM all-trans retinoic acid for 15 days. A 3D cell-printing system utilizing alginate, gelatin, and type I collagen was used to fabricate 3D Alginate bead scaffolds for the pre-macrophage and liver organoid interaction system[40].

Hepatobiliary organoids

The hiPSC line UC was selected and cultured in RPMI 1640 medium supplemented with 2% B27 minus insulin (B27-), activin A1 (100 ng/mL) and BMP4 (10 ng/mL) for 5 h[41,42]. The medium was then switched to 5% mouse Temin's serum replacement (mTeSR) (STEMCELL Technologies, Vancouver, BC, Canada) for 2 days, after which it was reverted to the previous medium for another 2 days. Subsequently, the medium was replaced with RPMI 1,640 containing 2% B27, 5% mTeSR, BMP2 (20 ng/mL), and FGF4 (30 ng/mL) for 5 days. Following this, BMP2 and FGF4 were substituted with HGF (20 ng/mL) and keratinocyte growth factor (KGF) (20 ng/mL) for an additional 6 days. Next, human cardiomyocyte maintenance (HCM) (Lonza, Basel, Switzerland) containing 10 ng/mL oncostatin M, 0.5 μM dexamethasone, and 10% cholesterol + MIX (ProbeChem, Shanghai, China) was applied for 20 days, after which the medium was changed to the same constituents, with the exception of dexamethasone. Notably, the optimal 10% cholesterol and MIX were the key elements to promote hepatobiliary-specific differentiation. The resulting

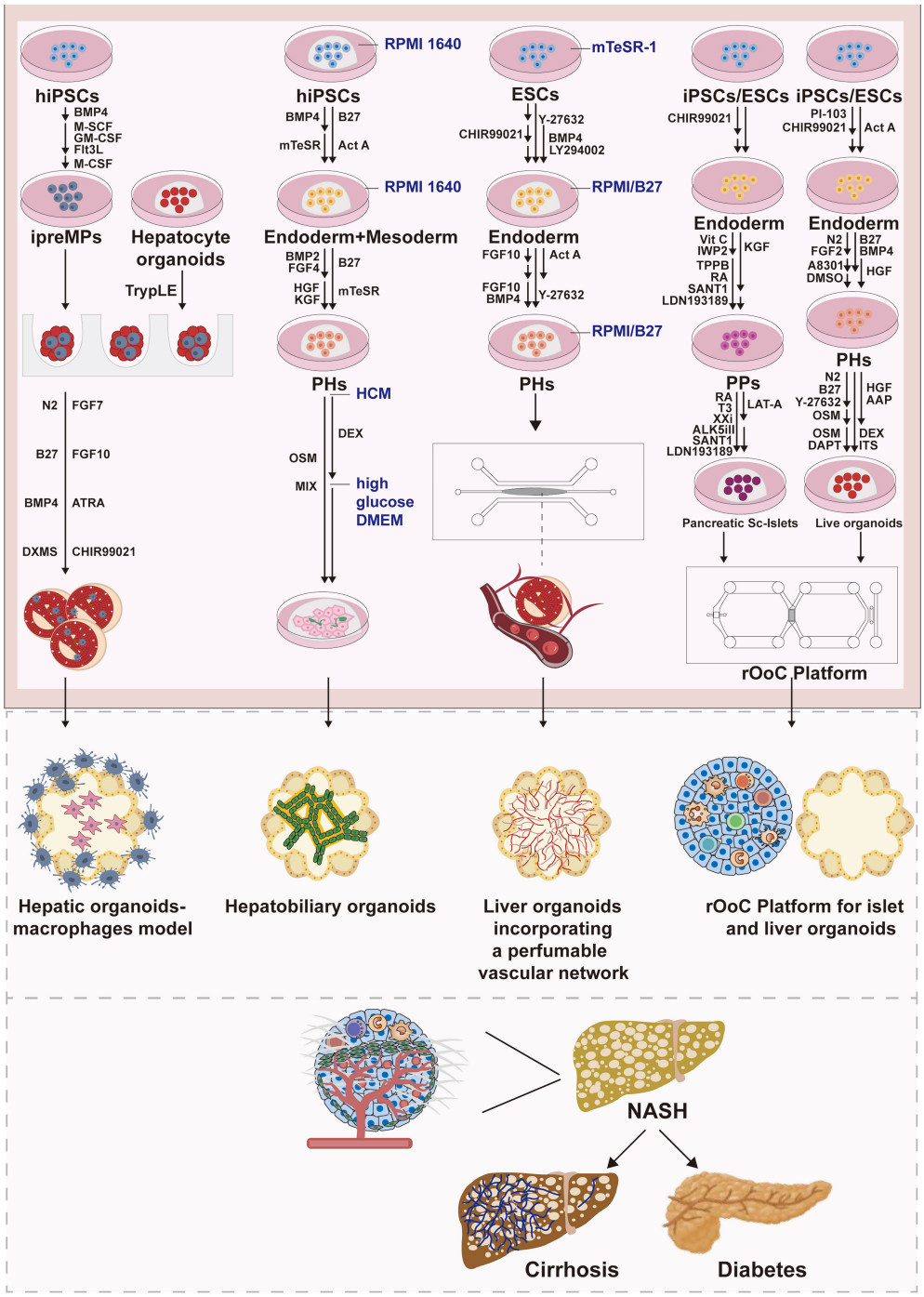

Figure 2. Multi-lineage liver organoids models such as hiPSC-derived 3D hepatic organoids-macrophages model, Hepatobiliary organoids, liver organoids incorporating a perfusable vascular network, and Organ-on-Chip Platform, for investigating MASLD progression. ESCs: Embryonic stem cells; hiPSC: human induced pluripotent stem cells; ipreMPs: human induced pluripotent stem cell-derived pre-macrophages; MASLD: metabolic dysfunction-associated steatotic liver disease; NASH: non-alcoholic steatohepatitis; rOoC Platform: Organ-on-Chip platform; PHs: primary hepatocytes; PPs: pancreatic progenitor cells; SC-islet: human induced pluripotent stem cell-derived pancreatic islet-like spheroid.

organoids exhibited a cystic structure characteristic of intrahepatic biliary epithelial cells, expressing key markers such as KRT19, CFTR, SSR2, ASBT, AE2, AQP1, and GGT1.

Liver organoids incorporating a perfusable vascular network

Cultured embryonic stem cells were used to induce differentiation into liver buds[43]. After incubation with Y-27632 (10 μM) and activin A (100 ng/mL), the cells were seeded onto Matrigel-coated plates and underwent a series of differentiation stages: definitive endoderm stage (Day 1-2: cultured in mTeSR-1 medium with Y-27632 and activin A; Day 2: additional supplementation with 10 ng/Ml BMP-4, 10 μM LY294002, 3 μM CHIR99021; Day 3: further addition of 10 ng/Ml BMP-4 and 10 μM LY294002); hepatic endoderm specification stage (Day 4: cultured in RPMI/B27 medium with Y27632, activin A, and 10 ng/mL FGF-10; Day 5-7: Y27632 and activin A; Day 8: BMP4, FGF10, and Y-27632); and liver bud stage (Day 9-12: cultured in RPMI/B27 medium with BMP4 and FGF10). After the liver buds sprouted, cells expressing both the kinase insert domain receptor and CD31 were observed along the vascular networks of the organoids. To further mimic *in vivo* conditions, an organ-on-a-chip platform was used, consisting of three layers of PDMS: a cell culture layer, a stretching layer, and a pressure chamber layer. The cyclic stretching of this system enhanced the expression of epithelial-mesenchymal transition (EMT)-related genes, as well as HGF and MMP9, while activating mechanosensitive channels, such as PIEZO1 and TRPV4, which contribute to vascular development and related signaling pathways.

rOoC Platform

Insulin resistance, obesity, and MASLD are disorders with reciprocal causation[4]. To study the cross-talk between liver and islets, a bidirectional communication system between islet and liver organoid compartments has been established through directed flow across two perfusion channels. A permeable membrane serves as a barrier between two organoids, allowing for molecular communication. The most key feature of this platform is that the insulin required for liver organoids is totally provided by the islets on the chip. This Organ-on-Chip (rOoC) Platform maintains glucose concentrations of 5-7 mM, almost the same as the physiological levels in human blood. Meanwhile, the glucagon secretion from the islet organoids was approximately 50-60 pmol, close to the 20-40 pmol range observed in humans.

These multi-lineage liver organoids can be used to investigate the mechanisms behind MASLD progression to liver cirrhosis and diabetes. Genetic variants (e.g., glycerol-3-phosphate acyltransferase, mitochondrial genes, and chromosome 2 open reading frame 16), hepatocyte-derived extracellular vesicles, and organokines (e.g., fetuin-A and adiponectin) collectively promote the progression of MASLD to diabetes[44].

Discovering novel mechanisms underlying MASLD [Figure 3]

Uncovers phenotype heterogeneity triggered by different modeling conditions

Exposure of the liver to excessive free fatty acids, including oleic acid (OA) and palmitic acid (PA), induces high levels of intrahepatic triglycerides infiltration, which subsequently damages cell organelles, including mitochondria and cell membrane, and triggers inflammation pathways that recruit multiple immune cells. These pathways promote the secretion of inflammatory mediators, such as TGF-β, which greatly promotes the transdifferentiation of HSCs into myofibroblasts and leads to excessive extracellular matrix deposition. This process has been recognized as a key aspect of liver inflammation and fibrosis during the progression of MASLD to NASH and related fibrosis. Single-cell transcriptomics analysis of hiPSCs-derived liver organoids reveals distinct transcriptional landscapes when stimulated by TGF-β1, OA, and PA, respectively[37]. At the cell-cell interaction level, the relative induction of interactions increased stepwise from OA- to PA- and finally to TGF-β1-treated liver organoids. OA moderately increased the interplay between hepatoblast 2-, cholangiocyte-, and dendritic-like cells, while PA elevated cross-talk among cholangiocyte-, hepatocyte progenitor-, fibroblast-, and myofibroblasts-like cells. However, TGF-β1 mediated high levels of interaction between myofibroblast-like cells and HSCs. In terms of molecular binding patterns, TGF-β1-treated liver organoids predominantly presented a receptor-ligand expression model involving PDGFB and PDGFRB, whereas the CXL2 and DPP4 pair expression pattern was

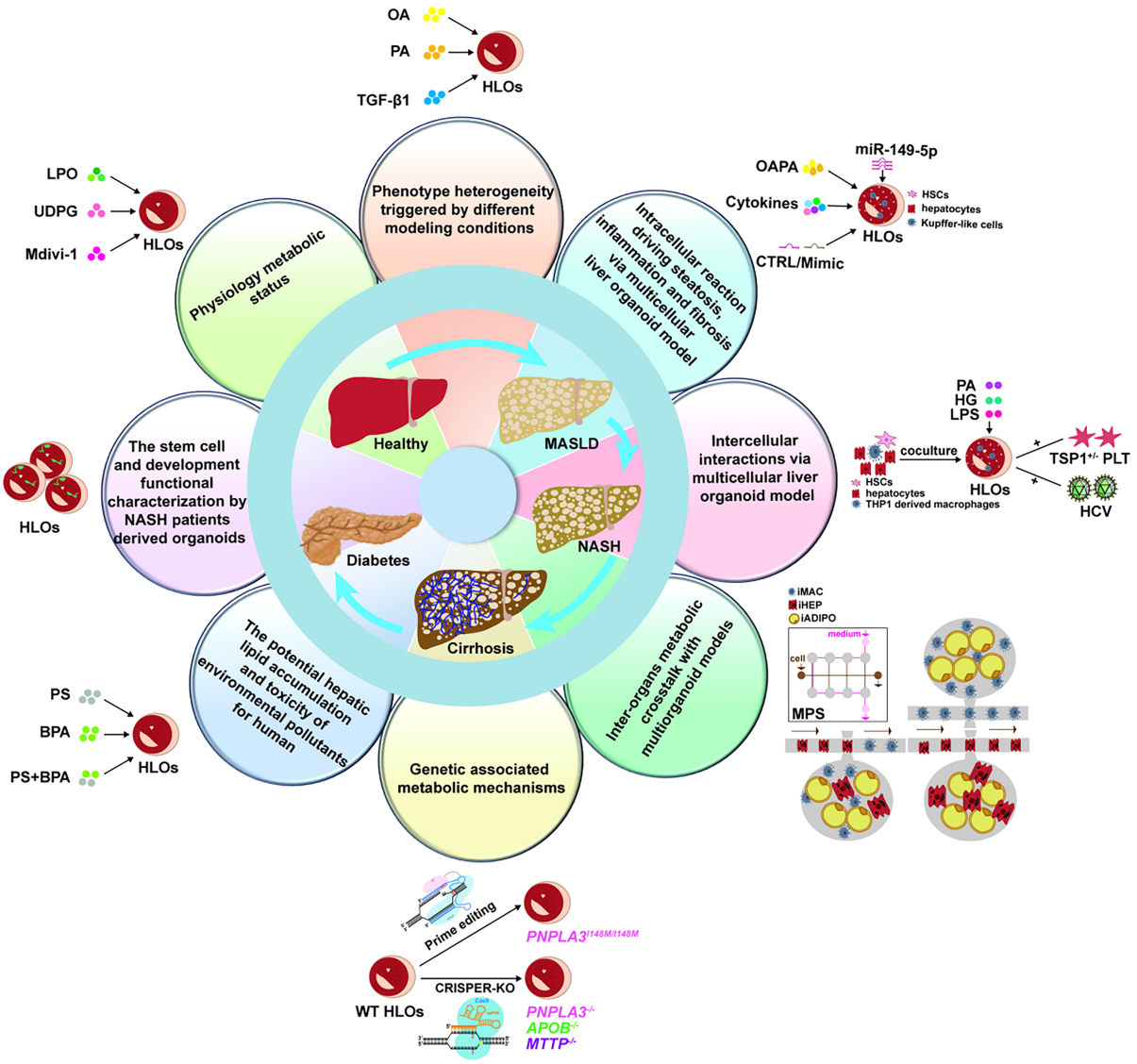

Figure 3. Applications of liver organoids in MASLD progression: (1) uncover phenotype heterogeneity induced by different modeling conditions such as lipid, glucose, and other metabolites; (2) identify intracellular reaction driving steatosis, inflammation, and fibrosis; (3) Identify intercellular interactions among hepatocytes, macrophages, Liver Sinusoidal Endothelial Cells, and Hepatic Stellate Cells; (4) Reveal inter-organs metabolic cross-talk; (5) indicate genetic-associated metabolic mechanisms via gene-edited cell lines-derived liver organoid models; (6) implicate the potential hepatic lipid accumulation and toxicity of environmental pollutants in humans; (7) unveil the stem cell function and developmental characterization in MASLD; (8) Mimic physiological metabolic status. BPA: Bisphenol A; CTRL: control; HCV: hepatitis C virus; HLOs: human liver organoids; HG: high glucose; HSC: hepatic stellate cell; iADIPOs: iPSC-derived adipocytes; iHEPs: iPSC-derived hepatocytes; iMACs: iPSC-derived macrophages; LPO: lactate, pyruvate, and octanoic acid; LPS: Lipopolysaccharide; MASLD: metabolic dysfunction-associated steatotic liver disease; MPS: microphysiological system; NASH: non-alcoholic steatohepatitis; OA: oleic acid; OAPA: oleic:palmitic acid; PA: palmitic acid; PLT: platelet; PS: polystyrene; TSP1: thrombospondin 1; UDPG: uridine diphosphate glucose; WT: wild type.

predominant in OA-treated organoids, and the TNF superfamily (TNFS) signatures were identified in PA-treated models. Further gene expression analysis showed that OA treatment enhanced the transcription of AFP, APOA1, APOC1, HNF4A, and CEBPA, which are associated with hepatocyte precursor proliferation, but TGFBI and TIMP1 were enriched in TGF-β1-treated DC-like states. These findings indicate that different model-building methods might only recapitulate one of the various hallmarks of MASLD progression in a dynamic, multi-lineage human *in vitro* system.

Identifying intracellular reactions driving steatosis, inflammation, and fibrosis via a multicellular liver organoid model

A multicellular liver organoids model, comprising hepatocytes, Kupffer-like cells, and HSCs and mimicking *in vivo* environments *in vitro*, enables standardization assessments of target molecule functions and phenotypes, although non-tissue-specific effects may still occur after modulating gene expression via transgenic vectors in murine models. A recent study demonstrated that hepatic miR-149-5p regulates whole-body metabolism and lipid utilization in mice with MASLD. The authors further used hiPSCs-derived organoids to testify that overexpression of miR-149-5p increased lipid accumulation in hepatocytes, as well as mild inflammation and fibrosis marker expression[45]. In situations where a target protein might serve opposite roles in steatosis, inflammation, and fibrosis, using organoid models with different cocultured cell types may provide novel insights into genotype-phenotype associations. By applying human-based organoids to evaluate liver-specific phenotypes in relation to overall metabolic status, it was found that the glucokinase regulatory protein (GCKR)-rs1260326: C>T aggravates MASLD severity in diabetes while protecting against fibrosis development under non-diabetic status[46].

Identifying intercellular interactions via multicellular liver organoid model

A commonly used model for studying intercellular interactions is the transwell coculture system. However, this system lacks advanced tissue structures and cannot replicate tight junctions between different cells or account for the mediating effects of the extracellular matrix. The *in vitro* 3D human NASH organoid model consists of at least three cell types: hepatocytes, THP-1-derived macrophages, and HSCs. This model forms 3D spheroids, which offer convincing evidence linking circulating cell-derived markers, such as platelet-derived TSP1, to the upregulation of inflammation and fibrosis signaling pathway genes[47]. However, one major challenge in using multicellular organoid models for MASLD is determining the optimal cell mixture ratio before experiments begin. A pioneering study did not detect differences in hepatocyte albumin levels when iPSCs-derived hepatocytes and iPSCs-derived macrophages were mixed at various ratios[48]. However, single-cell RNA sequencing indicated that coculture with iPSCs-derived macrophages did lead to a shift in the expression of macrophage receptor with collagenous structure (MARCO) and activated matrix collagen markers in HSCs.

Revealing inter-organ metabolic cros-stalk with multi-organoid models

Chronic inflammation in white adipose tissue (WAT) has been recognized as a critical early event in the pathogenesis of MASLD-related liver insulin resistance[49]. Previous studies have been limited to murine models and correlation analyses of patient samples. Recent advancements in organoid technology have enabled the creation of microphysiological systems using iPSC-derived hepatocytes, adipocytes (iADIPOs), and macrophages (iMACs) through a transwell coculture system[50]. By using fat and liver tissues derived from NASH patients, it was observed that activated, inflamed iMACs could induce insulin resistance in iHEPs, independent of steatosis, after transwell coculture. The coculture of tissue-resident activated macrophages with iADIPOs presented decreased fatty acid uptake compared to iADIPOs cultured alone without iMACs.

Indicating genetic-associated metabolic mechanisms

Genetic backgrounds accounted for the intrinsic differences in susceptibility to MASLD and its progression. Patient-derived liver organoid models, created from isolated cells, allow for the study of liver changes without the interference of gene editing. Moreover, organoids serve as models for investigating sex differences and the specific effects of single-locus mutations. The PNPLA3 I148M variant is a well-recognized gene polymorphism, though its underlying mechanisms remain unclear. In a study using primary liver cells (PLCs)-based organoids from males and females with MASLD, exposure to an estrogen

receptor-α agonist led to different degrees of upregulation ofPNPLA3 mRNA levels, which provides indirect evidence of a link between estrogen receptor-α and a PNPLA3 enhancer site[51]. Another report utilized iPSC-liver organoids harboring mutations in the *DKC1* gene to replicate the characteristics of MASLD, including steatosis, nodular hyperplasia, hepatitis, and cirrhosis. Additionally, hepatostellate organoids combined with single-cell transcriptome analysis confirmed that the abnormal activation of the Notch signaling pathway in endothelial cells was responsible for the worsening of liver fibrosis. Of note, pilot studies have compared the differences between liver organoid models treated with extrinsic lipid acids and those generated using CRISPR-engineering techniques[52]. These studies showed that free fatty acids significantly attenuated organoid proliferation, a phenomenon not observed in APOB- and MTTP-mutant models. This finding provides new insights, suggesting that de novo lipogenesis-induced organoids may reveal distinct aspects of MASLD.

Potential hepatic lipid accumulation and toxicity of environmental pollutants in humans

The effects of environmental pollutants on health can vary between humans and other animals, even at the same exposure levels, due to interspecies differences. Liver organoid models overcome the challenge of obtaining living human liver tissues, and offer a promising tool that more closely replicates the hepatic immune environment, enabling the identification of human-specific changes triggered by contaminants. Studies using PSC-derived liver organoids[53] have identified polystyrene microplastics and the plasticizer bisphenol A as inducers of liver lipogenesis markers (SCD1, ACOX1, ESR2, and CEBPB), suggesting their potential role in oxidative stress and inflammation associated with MASLD.

Unveiling stem cell function and developmental characterization using NASH patients-derived organoids

Establishing organoids from primary liver stem cells obtained directly from end-stage cirrhotic livers of patients with NASH might help understand individual differences and facilitate drug efficacy testing. A recent study successfully generated bipotent ductal organoids from liver samples (50-100 mg) collected from liver transplant recipients[54]. Findings revealed that NASH-derived organoids exhibited significantly prolonged growth times for hepatocytes and biliary cells - up to 2 weeks longer than those of healthy controls to reach comparable sizes. Additionally, they displayed a higher percentage of irregular shapes and an impaired ability to reenter the proliferative state. Changes in biliary epithelial cell activation might be related to E2F transcription factors, which are mediated by lipid overload in organoids[55]. Compared to Hu7 cell lines and healthy organoid controls, NASH organoids demonstrated greater apoptosis sensitivity when exposed to the same levels of free fatty acids. Metabolic analysis indicated decreased albumin secretion and low-density lipoprotein (LDL) uptake (reduced by approximately twofold), but increased lipid accumulation, elevated CYP3A4 activity, and varied ammonia degradation. The heightened functional sensitivity of hepatic organoids might offer a useful tool for identifying phenotypes related to target molecules.

Mimicking physiological metabolic status

Traditional immortalized cell lines may undergo metabolic adaptions due to the continuous maintenance of reproductive conditions, which lack psychological rhythms such as long-term insulin stimulation. Additionally, herbivorous and primate animals have inherently different dietary habits, leading to the evolution of distinct metabolic regulatory mechanisms. The passage and large-scale biofabrication of liver organoids make it possible to assess and validate metabolic mechanisms and drug efficacy in MASLD. Studies have shown that human liver organoids exhibit significantly greater excessive lipid accumulation than murine-derived organoids when exposed to low levels of lactate, pyruvate, and octanoic acid[56]. A recent study using PSCs-derived organoids found that stimulation with the glycogenesis metabolite uridine diphosphate glucose (UDPG) facilitates its transport to the Golgi apparatus to block the protease S1P, thus

inhibiting the cleavage of sterol regulatory element-binding protein (SREBP), thereby inhibiting lipogenesis in MASLD[57]. Additionally, variations in metabolite abundance can serve as indicators of specific disease stages in differentiated organoids. For example, mass spectrometry analysis has identified higher levels of oxysterols secreted by NASH organoids, which may act as early-stage biomarkers for liver fibrosis initiation[58].

Mitochondria, the central subcellular organelles in metabolism, exhibit dynamic changes in morphology, including fission, fusion, and autophagy. In a methionine-choline-deficient diet mouse model, NASH-derived organoids displayed a higher prevalence of swollen and spherical mitochondria. Treatment with mitochondrial division inhibitor 1 was shown to rescue these abnormal mitochondrial dynamics and mitigate related inflammation and fibrosis[59].

In summary, we provide a detailed overview of various modalities developed in the culture and application of mini-liver organoids derived from different PSCs. These organoids represent a revolutionized frontier tool for examining intracellular, intercellular, and inter-organ connections. Their humanized traits enable the identification of target genes that more closely align with human physiology and liver-specific phenomena. They also hold promise for bridging the gap between co-morbid metabolic diseases, pre-MASLD, and MASLD with the advancement of multi-organoid *in vitro* systems[60]. However, several challenges hinder the broader application of these tools, including the lack of liver zone-specific conditions such as circulating oxygen, cytokines, and metabolite gradients. Furthermore, there have been no reported integrations of neural or intestinal systems into liver organoids, limiting our understanding of the liver-brain-gut axis. Despite these limitations, organoid platforms provide an essential and irreplaceable basis for further functional and mechanistic studies of MASLD, offering significant advantages over traditional cell lines and murine models. Moving forward, further optimization is needed to enhance liver organoids with more humanized organotypic characteristics.

DECLARATIONS

Authors' contributions

Equally participated in literature search, manuscript writing, and editing: Feng T, Li J
Wrote and reviewed the manuscript, and provided insightful comments: Ye J
Supervised the literature search, discussion, and writing process: Ma X
All authors carefully read and approved the final manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

Ye J is a Youth Editorial Board member of the journal *Metabolism and Target Organ Damage*. Ye J was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, and decision making. The other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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