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WORD COUNT	4978	TIME SUBMITTED	19-MAR-2024 03:13PM
		PAPER ID	107669645

Name of Journal: *World Journal of Gastroenterology*

Manuscript NO: 91833

Manuscript Type: REVIEW

Necroptosis contributes to non-alcoholic fatty liver disease pathoetiology with promising diagnostic and therapeutic functions

Sun HJ *et al*. Necroptosis in NAFLD

Abstract

Lesions of the ampulla of Vater represent an uncommon group of gastrointestinal malignancies. The majority of lesions of the ampulla of Vater are either adenomas or adenocarcinomas. Ampullary lesions are often incidental findings. Accurate preoperative diagnosis and staging of ampullary tumors is imperative for predicting prognosis and determining the most appropriate therapeutic approach. Endoscopic ampullectomy is a safe and efficacious therapeutic procedure that can obviate the need for potentially major surgical intervention. This review will provide the framework for the diagnosis and management of ampullary lesions from the perspective of the practicing gastroenterologist. Strategies for safe and successful endoscopic ampullectomy with a focus on accurate preoperative diagnosis and staging, resection technique, and management of complications are presented.

Key words: Ampullary adenoma; Papillary tumors; Endoscopic ampullectomy; Endoscopic ultrasound; Pancreatitis

Sun HJ, Jiao B, Wang Y, Zhang YH, Chen G, Wang ZX, Zhao H, Xie Q, Song XH. Necroptosis contributes to non-alcoholic fatty liver disease pathoetiology with promising diagnostic and therapeutic functions. *World J Gastroenterol* 2024; In press

Core tip: Adenomatous ampullary lesions are rare. Endoscopic retrograde cholangiopancreatography and endoscopic ultrasound (EUS) have changed the management of patients with these lesions. Endoscopic ampullectomy is a technique that has revolutionized the treatment of these lesions avoiding potential complications of surgery. We herein discuss the epidemiology, the role of EUS in the local staging and the role of endoscopy in the treatment of the adenomatous ampullary neoplasms.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is described as the most prevalent type of chronic liver disease worldwide[1]. NAFLD is considered a growing cause of end-stage hepatic disorders throughout the world and emerged as a pathoetiology of hepatocellular carcinoma (HCC) even when the underlying cirrhosis is absent[2,3]. In fact, the excessive accumulation of lipids in the hepatocytes (in the form of triglycerides, > 5% fat content in the liver; referred to as steatosis) of people consuming alcohol at low-risk amounts is the main characteristic of patients with NAFLD[4]. Clinically, the condition may be restricted to excessive liver fat, known as NAFL, or progress to necroinflammation and fibrosis, called non-alcoholic steatohepatitis (NASH), to NASH-cirrhosis, and eventually to HCC[5,6]. In Western countries, it is estimated that one-third of the general population is affected by NAFLD which is associated with excess body weight and diabetes mellitus. Moreover, the disease is highly prevalent in the Middle East and the rate of incidence is growing in the Asian subcontinent and the Far East nations[7-9]. Altogether, NAFLD has become the most common chronic liver disorder with a worldwide prevalence of around 25% of the adult population that is recognized to be closely and bidirectionally related to components of metabolic syndrome[9,10].

The most important challenge is the identification of people with NAFLD who are at the highest risk of developing liver-related complications. The burden of end-stage liver disease is estimated to increase two to three times globally by 2030[11]. Although NALFD is clinically accepted as the most rapidly increasing cause of liver-related mortality emerged as a significant cause of end-stage liver disease, HCC, and liver transplantation with a substantial health economic burden, NAFLD is underappreciated as a remarkable chronic disorder and there is a few numbers of managing strategies or policies[12,13]. In addition, even though a variety of ongoing studies have assumed several

genetic/metabolic aberrations as the causes of NAFLD pathogenesis, the underlying mechanism by which the disease occurs and progresses remains unclear, making early laboratory diagnosis and effective treatment challenging[14-17].

Generally, a variety of unrecoverable intra- or extra-cellular perturbations capable of disrupting cellular survival affect cells by the activation of one of many signaling cascades, causing the death of cells and tissue damage[18-20]. The gross diversity in cell death programs has made these processes fall into two major categories including accidental cell death, and regulated cell death (RCD). Accidental cell death is described as a passive process in which uninspected necrosis is the main type, while RCD is an active process that includes a number of subtypes[18-21]. Specific molecular mechanisms are considered the initiating and propagating agents of RCD modalities with remarkable interactivity. In addition, each type of RCD, discussed later, represents distinct molecular, biochemical, functional, and morphological properties with particular pathophysiological consequences. Two main categories of RCDs consist of apoptotic and non-apoptotic cell death programs, which include apoptosis in the first subtype and necroptosis, pyroptosis, autophagy, and ferroptosis in the second one[18-20,22]. Fortunately, a large number of studies have recently focused on determining the process of hepatocyte cell death related to NAFLD in order to elucidate the etiology of the disease and suggest effective therapeutic and diagnostic options[23]. Considering the breadth and complexity of the findings, the present study has aimed to first provide an overview of the types of RCDs and the contribution of each one in the disease progression briefly, and then discuss in detail the role of necroptosis, a novel form of RCD, in the pathoetiology and treatment of the disease.

RCDs contribute to the progression of NAFLD

RCD is considered intrinsically associated with inflammatory disorders of hepatic tissue and is documented to be pivotal in governing the clinical consequences of liver disorder[24,25]. A plethora of evidence has revealed different forms of RCD pathways with increasingly identified correlations with NAFLD[23]. Significantly, the novel described forms of RCDs may co-exist simultaneously in diseases, and a number of them portion overlapping molecular processes that may function as a backup dying approach to provide the survival of an organism when a cellular threshold induced by death is established[26-28]. In fact, hepatocellular death could be triggered by metabolic, viral, toxic, and/or autoimmune mediators accompanied by inflammation and compensatory proliferation which frequently represent a close association with the development of cirrhosis, fibrosis, and HCC[29].

Conventionally, apoptosis was described as a strictly controlled pathway, as opposed to the passive form of cell death known as necrosis which is an irregulated and accidental form of cellular death[30]. The external forces cause irreparably cell injury the passive form of cell death occurs which is characterized by oncosis, a rapid organelle, and cytoplasmic swelling[31]. Moreover, cell membrane permeabilization followed by leakage of damage-associated molecular patterns (DAMPs) occurs that initiates the immune response[32]. Recently, a plethora of research highlights a variety of forms of RCD modalities such as autophagy, ferroptosis, pyroptosis, and necroptosis that represent similar main morphological characteristics with necrosis[33,34], however, are regulated molecular pathways and have well-defined processes (Table 1).

Necroptosis represents several similar molecular components with apoptosis, particularly the extrinsic pathway, hence it may be the best-understood form of regulated necrosis. Also, necroptosis provides the progression of cellular death when apoptosis is pathologically inhibited, which in turn could be assumed as a disease state in the hepatic tissue[4]. Nonetheless, other mentioned

forms of RCD probably be significant in the progression of NAFLD as a proper characterization of RCD in the disease can lead to promising diagnostic and therapeutic options. The novelty of the field has led to the rapid progress of research, and recent studies sought to describe the association between the non-apoptotic forms of RCD and the progression of NAFLD. Although the focus of the current study has been on elucidating the contribution of necroptosis in the pathogenesis of NAFLD, it is essential to first acquainted with other forms of non-apoptotic RCDs and briefly discuss the involvement of each one in the development of the disease.

An overview of pyroptosis

Zychlinsky *et al*[35] initially reported pyroptosis in the 1990s and described a lytic form of cell death in macrophages infected by *Shigella flexneri*. Nevertheless, the 'pyroptosis' term emerged in 2001 as scientists revealed that the death of macrophages induced by *Salmonella* was dependent on caspase (CASP)-1[36]. Subsequently, the number of pyroptosis-related CASPs that are opposed to apoptosis-related CASPs has significantly elevated and includes CASP-1, CASP-11, and the human orthologs CASP-4 and CASP-5[35,36]. Surprisingly the well-known apoptotic effector CASP-3 is considered a pyroptotic CASP under specific conditions[37,38]. Primarily, this type of RCD occurs after intracellular pathogens cause infection leading to the formation of cell membrane pores dependent on CASP activity, swelling, cell rupture, and release of pro-inflammatory interleukins[39] such as interleukin (IL)-1β and IL-18[40-42].

The pore-forming gasdermin D (GSDMD) was characterized in 2015 contributing as the executioner of pyroptosis[43,44]. 31-KDa N-terminal GSDMDNT fragment is produced when GSDMD is cleaved by the action of CASP-1 and CASP-11. GSDMDNT exhibits intrinsic pore-forming activity. Moreover, the cleavage of GSDMD produces a 22-KDa C-terminal GSDMDCT

fragment. This fragment attaches to GSDMDNT in order to inhibit the protein[43,44]. The upregulation of GSDMDNT alone is followed by the induction of pyroptosis, however, GSDMDCT blocks GSDMDNT-induced pyroptosis[43,44]. Importantly, it is documented that GSDMD belongs to a larger family of proteins consisting of GSDMA to GSDME (also known as DFNA5), and DFNB59[45]. Recent investigations revealed that GSDME functions as another pyroptosis executioner, that is capable of switching CASP-3-mediated apoptosis to pyroptosis[46,47]. The majority of GSDMs have been linked to the incidence and development of a variety of diseases, however, the exact molecular and functional activation mechanisms remain mainly unknown[48].

Two signaling pathways including canonical and non-canonical signalings activate pyroptosis. These two signalings differ in the application of cytoplasmic multiprotein complexes known as inflammasomes[49,50]. Inflammasomes trigger the canonical pathway of pyroptosis. In this regard, inflammasomes can recognize different endogenous and exogenous danger signals such as pathogen-associated molecular patterns (PAMPs) and DAMPs. The canonical inflammasomes consist of a sensor protein that belongs to the nucleotide-binding domain (NBD), apoptosis-associated speck-like protein containing a CARD (ASC), AIM2-like receptor or NLR (leucine-rich-repeat-containing) or pyrin family, and pro-CASP-1 which is an inactive zymogen[51]. When the canonical inflammasome is formed, the activation of CASP-1 leads to the cleavage of pro-IL-1β and pro-IL-18 into their active forms. Next, these two ILs are released extracellularly subsequent to the action of GSDMDNT that causes spore formation in the cell membrane. Whereas, the non-canonical pathway is CASP-11-dependent without inflammasome priming cleaves GSDMD. In this type of pyroptosis, GSDMDNT signals back to canonical NLRP3 inflammasome leading to the activation of the CASP-1-dependent pathway[52].

Pyroptosis may contribute to NAFLD progression and transition to NASH

A variety of factors such as lipotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress, hepatocyte death pathways, and innate immune response are able to initiate chronic inflammatory processes in the liver that may provide fuel for the transition from NAFL to NASH[53-55]. The infiltration of macrophages and activation of local Kupffer cells is considered a key characteristic of disease pathoetiology[56]. Tumor necrosis factor (TNF)-α is released by Kupffer cells[57] which feeds a vicious cycle of inflammatory responses and initiates fibrosis after activating apoptosis. Nonetheless, it has recently appeared that inflammatory CASPs such as human CASP-4/5, CASP-1, and murine CASP-11 contribute pivotally as inflammation mediators[44]. Thereby, it is assumed that pyroptosis is crucially involved in NAFL development and progression to NASH.

It is documented that the activation of inflammasome by typical factors such as uric acid, DAMPs, and fatty acids, which increase the expression of NLRP3 components, could trigger the activity of CASPs, promoting inflammation, and causing liver fibrosis[58]. The excessive function of inflammation-related CASPs is implicated directly in the NAFLD pathoetiology, where key effector molecules are considered to be pro-inflammatory cytokines released meanwhile[59,60]. Moreover, the generic substrate for inflammatory CASPs, GSDMD, functions as a downstream mediator of the activation of non-canonical inflammasome by contributing to inflammatory CASP-mediated pyroptosis[61,62]. Importantly, the GSDMDNT domain representing intrinsic pyroptosis-inducing activity in patients with NASH was positively associated with the NAFLD activity score and fibrosis[63]. In fact, the lipogenic gene *Srebp1c* downregulation and upregulation of *Ppara*, a lipolytic gene, and its downstream targets, induced the protection of *Gsdmd*-/- animals from steatosis[63].

As mentioned earlier, DAMPs and PAMPs can cause pyroptosis-related hepatocyte death directly or indirectly causing hepatic damage. It is reported that animals with mutations in myeloid-specific *Nlrp3* do not reveal detectable pyroptotic-mediated hepatocyte death and represent less severe hematopoietic stem cell activation[64]. Hence, one can conclude that in addition to immune cells, pyroptosis in hepatocytes caused by the activation of intrinsic inflammasome can exacerbate inflammation and fibrosis in the liver, determining that both immune cell- and liver-specific NLRP3 inflammasome activation as essential contributors to liver injury[64,65]. However, it is required to investigate hepatocyte-specific *NLRP3* mutant animals to provide convincing evidence of the correlation between hepatocytes and pyroptosis in the onset and progression of liver injury in NAFLD.

An overview of ferroptosis

Ferroptosis was initially reported early 20th century by a cell-permeable compound called erastin, a compound which was lethal to cancer cells of humans with an oncogenic *RAS* mutation[66]. A decade later the term ferroptosis was established to describe an erastin-caused RCD mediated by the accumulation of lipid peroxides dependent on iron[67]. Ferroptosis exerts tumor-suppressor activities, increased mitochondrial membrane density, and cell shrinkage without any typical necrotic or apoptotic manifestations[67]. Similar to pyroptosis, two different signalings including canonical and noncanonical pathways are described as ferroptosis inducers. In the canonical pathway, the glutathione (GSH) peroxidase 4 (GPX4) enzyme is inactivated eighter directly or indirectly which induces ferroptosis, whereas, in a noncanonical manner, the labile iron pool is increased[67].

The direct interaction of erastin with the transporter solute carrier family 7 member 5 (SLC7A5) and subsequent disruption of amino acids transport into the cell by the Xc- system occurs in the canonical pathway of ferroptosis[67]. The

regulatory SLC3A2 and a catalytic subunit SLC7A11 are components of the Xc- system that are responsible for the exchange of cystine with glutamate by elevated promotion of cystine cellular uptake[68]. Cystine is the plasma precursor of cysteine that is essential for the synthesis of GSH, a major redox regulatory system[69]. Therefore, the blockage of cystine by inhibitors (*e.g.*, erastin, L-glutamate, *etc.*) is followed by the inhibition of GSH synthesis, suppression of GPX4, and accumulation of phospholipid hydroperoxides (PL-OOH), considered the main mediator of lipoxygenases (LOXs) chain reactions[70].

In the state of intracellular free iron overload, it interacts with reactive oxygen species[71] which ultimately leads to the production of hydroxyl radical that is highly reactive to macromolecules such as polyunsaturated fatty acids (PUFAs)[72]. The oxidation of PUFAs *via* a pathway involving lysophosphatidylcholine acyltransferase 3, acyl-CoA synthetase long-chain family member 4, and LOXs is required for ferroptosis caused lipotoxicity[73-75]. GPX4 is considered the only member capable of reducing membrane phospholipid hydroperoxides determining its significant contribution to confronting permeabilization of plasma membrane, peroxidation of lipids, and ultimately release of DAMPs[76]. In the non-canonical pathway, oxidative damage and ferroptosis are promoted by elevated uptake of iron by transferrin receptor and reduced export of iron by ferroportin[77,78].

Scarce information on the ferroptosis involvement in NAFLD progression

Unfortunately, there is scarce evidence demonstrating the contribution of ferroptosis in NAFLD pathogenesis. However, malondialdehyde and 4-hydroxynonenal[71], as secondary lipid peroxidation products, are suggested as stress markers in patients with NASH[79]. In this regard, well-known antioxidants capable of suppressing lipid peroxidation such as vitamin E and quercetin[80,81] potentially could reduce the levels of alanine transferase in

patients with NASH[82,83]. In addition, the accumulation of iron due to metabolic dysfunction is followed by the aggravation of NASH as liver cirrhosis was reported[39]. Similarly, the exacerbation of primary hemochromatosis is observed in patients with NASH and iron overload[84], while the removal of iron was accompanied by the amelioration of hepatic damage and alanine transferase levels[85]. Furthermore, evidence suggesting the role of ferroptosis in liver steatosis has been discussed[86,87]. Nevertheless, documented information regarding the role of ferroptosis in NAFLD deserves further investigation in appropriate patient models with the disorder, particularly since currently no exact therapeutic strategies are available.

The modification of ferroptosis is considered a novel therapeutic option to confront malignancies[88]. Tyrosine kinase inhibitors (TKIs) are described as the first approved systemic treatments for advanced HCC, however, the systemic treatment of HCC has been further developed with the immune checkpoint inhibitor[89]. Recent evidence suggests the treatment with atezolizumab plus bevacizumab over sorafenib. Fortunately, the latest findings have suggested novel therapeutic strategies based on RCD modifications to confront HCC[89,90]. Lenvatinib, a well-known TKI, could suppress HCC progression *via* the induction of ferroptosis through the inhibition of fibroblast growth factor receptor-4[91]. Metronomic capecitabine, as another example, has been suggested as a second-line therapy in HCC patients after sorafenib failure[92] or discontinuation[93]. Similarly, the study conducted by Wang *et al*[94] indicated the ability of metronomic capecitabine to induce ferroptosis in CD4+ T cells, which is probably attributed to autophagy-related GPX4 degradation in these immune cells, caused the amelioration of liver transplantation rejection[94]. Concordantly, artesunate is considered a well-tolerated and appropriate combination therapy that synergizes with sorafenib to promote ferroptosis in HCC cells[95]. Moreover, GSH S-transferase zeta 1, an enzyme involved in the catabolism of phenylalanine, can inhibit the NRF2/GPX4 axis leading to

sensitizes HCC cells to sorafenib-induced ferroptosis[96]. Similarly, tiliroside induces ferroptosis *via* targeting TANK-binding kinase 1 leading to the death of sorafenib-resistant HCC cells[97]. Therefore, it appears necessary for further studies to address the effects of ferroptosis modulators on the death of HCC cells treated with chemotherapeutics.

An overview of necroptosis

Ray and Pickup[98] provided the first evidence of necroptosis in 1996 when they observed a lytic mode of pig kidney cell death infected with the cowpox virus governed by the expression of a CASP inhibitor known as the viral cytokine response modifier A. Four years later, Holler *et al*[71] revealed that the classical death receptors including FAS, TRAIL, and TNF receptors triggered cell death by two alternative pathways. One of these pathways relied on CASP-8, the classical extrinsic pathway of apoptosis, while the one that was dependent on the receptor-interacting protein kinase 1 (RIPK1), the necroptosis. Nevertheless, it was in 2005 when this mode of cell death was named as Degterev *et al*[99] demonstrated that a compound that inhibits the kinase activity of RIPK1, known as necrostatin-1, could inhibit the death of TNF-treated cell lines. Subsequently, the two downstream core components of the necroptotic machinery have been identified that are RIPK3 and mixed lineage kinase domain-like pseudokinase (MLKL)[100-102]. Necroptosis is primarily initiated after infections and stressors such as chemotherapy or radiation and morphologically exhibits the characteristics of necrosis, for example in response to extreme external factors with loss of membrane integrity, elevated cell volume, swelling of organelles, and cellular collapse. In addition, necroptosis is followed by the release of DAMPs such as IL-1α, high-mobility group box 1, and IL-33[103,104]. Specific DAMPs related to necroptosis have not been documented so far, however, the release of DAMPs during necroptosis

provokes a severe inflammatory response associated with the development of several diseases[103,104].

The activation of death receptors (DRs) (*e.g.*, TRAIL-R, CD95, TNFR1) and the inactivation, inhibition, or absence of apoptosis signaling components are the two main preconditions for the initiation of necroptosis. In fact, the formation of the RIPK1/RIPK3 platform known as necrosome occurs upon the activation of DRs[20]. In the necrosome, RIPK1 and RIPK3 interact with critical RIP homotypic interaction motifs to adopt a hetero-amyloid structure[105]. Next, RIPK3 phosphorylates MLKL leading to the oligomerization and finally translocation of MLKL to the cell membrane[20]. Along with RIPK1, it is suggested that family proteins of casein kinase 1 as necrosome ingredients directly phosphorylate human RIPK3 to induce necroptosis[106]. Ultimately, pore formation in the cell membrane occurs upon MLKL translocation accompanied by the increment of permeability through activation of Ca^{2+} influx, metalloproteinase and A disintegrin, and phosphatidylserine externalization[107-109]. It is widely accepted that remarkable crosstalk regulations exist between necroptosis and apoptosis in DR-dependent cell death pathways[110], hence one cannot be activated without inhibiting the other. Concordantly, CASP-8 cleaves and inactivates RIPK3 and RIPK1 revealing that apoptosis initiation suppresses necroptosis[111], whereas the activity of RIPK3 determines whether cells die by necroptosis or apoptosis[111,112].

In addition to DRs, a variety of pathways such as nucleic acid sensors (*e.g.*, Z-DNA-binding protein 1, also known as DAI)[113], toll-like receptors (TLRs) such as TLR4 and TLR3[114], retinoic acid-inducible gene 1 protein[115], TNF[116], and adhesion receptors[117] could initiate necroptosis. However, these signaling pathways are frequently RIPK1-independent, although phosphorylation and activation of RIPK3 and MLKL are required[20]. It should be noted that a membrane remodeling and scission machinery known as the endosomal sorting complexes required for transport-III complexes can promote membrane

repair and thereby limit MLKL-mediated necroptosis[118]. The contribution of MLKL to the regulation of endosomal trafficking and extracellular vesicle generation reveals a delicate balance between membrane injury and repair determining the ultimate cell fate in necroptosis[119].

Necroptosis exerts a pivotal role in NAFLD

A plethora of evidence has considered an increased level of necroptosis in human cells as one of the main events in the progression of different pathological states including NAFLD, which is generally accompanied by an increase in the infiltration of immune cells and the induction of inflammation[120-122]. Therefore, necroptosis can be assumed as a potential therapeutic target. In addition, the detection of patients with NAFLD and distinguishing it from pathological states with similarities in clinical manifestations and laboratory findings may be another merit of examining necroptosis in patients. In the following, the current study first discusses the participation of necroptosis in the pathoetiology of the disease and then critically reviews the possible application of necroptosis as a novel diagnostic and therapeutic strategy.

Necroptosis contributes to the progression of the disease

Shreds of evidence during the last two decades have suggested that necroptosis is involved in the occurrence of the disease and actively participates in the progression of NAFLD towards NASH and HCC. The occurrence of cell death in hepatocytes is assumed to be necessary for the occurrence and progression of NAFLD[123,124]. Accordingly, it has been documented that NAFLD coincides with the induction of inflammation, disruption of lipid homeostasis, and characteristics of metabolic syndrome[125], meanwhile, necroptosis is involved with inflammatory responses and intracellular bioenergetic regulation[126,127]. In this regard, the involvement of the RIPK3 in the mitochondrial bioenergetics

of hepatocytes[128], the necroptotic death of white adipocytes in NAFLD patients[129], and the induction of necroptosis caused by oleic/palmitic acid imbalance in hepatocytes isolated from patients[130] with NAFLD could be assumed valid markers of the contribution of necroptosis to the lipid metabolism-dependent occurrence of NAFLD. In addition, the induction of necroptosis caused by inflammatory mediators such as TNF[131,132], TLR4[133], and IL-6[134] is considered another event involved in the occurrence of NAFLD.

In addition to inflammatory and metabolic mediators, other signaling pathways may initiate necroptosis and cause NAFLD occurrence. For instance, it has been demonstrated that polarity protein AF6 can directly interact with the intermediate domain of RIPK1 and regulate its ubiquitination mediated by the deubiquitylase enzyme USP21 leading to the promotion of necroptosis in hepatocytes[135]. In this regard, the overexpression of AF6 results in the TNFα-induced necroptosis-mediated mortality of liver cells while hepatocyte-specific deletion of AF6 suppressed necroptosis and the subsequent inflammation in different non-alcoholic liver diseases[135]. The prevention of NAFLD *via* restriction of MLKL-dependent necroptosis by epigenetic silencing of RIPK3 reveals that the initiation of necroptotic-mediated liver cell death contributes to the NAFLD occurrence[136]. In addition to necroptosis-related death, MLKL signaling is involved in NAFLD pathogenesis by regulating other cell death programs such as autophagic flux[137]. Forkhead box protein O1 (FOXO1) is another effective factor in inducing necroptosis and NAFLD where two distinct studies have covered this issue with a different approach. The first study by Qian *et al*[133] showed that Serpina3c deficiency induced necroptosis and NAFLD by FOXO1 overexpression, while the other assumed that FOXO1 induces necroptosis and endoplasmic reticulum stress, and as a result, is involved in the pathogenesis of the disease[138]. Similarly, it has been documented that necroptosis induced by oxidative stress plays a key role in the pathogenesis of NAFLD and subsequent liver fibrosis[139,140].

Disease progression towards NASH and HCC is one of the undesired consequences of necroptosis induction in NAFLD patients. Several studies have assumed that cross-talk between RCDs is involved in the promotion of inflammation and the establishment of NASH following NAFLD[87,137,141,142]. It has been repeatedly shown that the induction of inflammation (for example, through TNF and TLR) and the resulting necroptosis actively cause NAFLD-to-NASH transition[143,144]. Importantly, necroptosis has been described as a pathological event in the liver that facilitates the appearance of steatohepatitis, as it has been reported that RIPK1 and RIPK3 cause hepatocyte death and exacerbation of NASH by inducing inflammation within macrophages and interacting with the JNK pathway[145-147]. In addition, diet is one of the factors that may contribute to NAFLD progression and steatosis by modulating RIPK3, inflammation, and necroptosis[148,149]. Although rare evidence of necroptosis involvement in the NAFLD-to-HCC transition has been reported, two recent studies have clarified that RIPK3 as a regulator of lipid metabolism participates in liver carcinogenesis, and RIPK3/MLKL absence reduces the risk of carcinogenesis[150,151].

The active involvement of necroptosis in the occurrence and progression of the disease continues with its effect in determining the severity of hepatic tissue injury. For example, the exacerbation of liver injury due to myeloid deficiency of CCN3 is mediated through the activation of necroptosis[152]. Also, the crosstalk between necroptosis and inflammatory mediators promoted both necroptosis and inflammation in liver fatty cells and as a result, aggravated liver damage in NAFLD models[134,153,154]. SPARC overexpression is another pathological event that leads to severe liver damage in patients with NAFLD by increasing the level of RIPK1/RIPK3 and promoting necroptosis[155]. Importantly, the involvement of necroptosis in the pathogenesis of the disease as well as the aggravation of pathological consequences can promise it as a potential biomarker for NAFLD early detection, grading, and prediction of

progression. Moreover, targeting upstream effectors that promote necroptosis is an interesting novel strategy that may be effective in disease management. Therefore, in the next two sections, the findings related to the diagnostic and therapeutic efficacy of necroptosis in patients with NAFLD have been reviewed (Table 2).

The diagnostic value of necroptosis

Although the involvement of necroptosis in the occurrence of NAFLD and its progression to NASH and HCC has been appropriately elucidated, rare reports of the diagnostic value of necroptosis in the early detection and grading of the disease have been presented. Considering the coincidence of necroptosis and inflammation and the strengthening effect they have on each other, the main markers presented have been related to inflammatory responses. For example, the evidence presented on the changed levels of TNF-α, IL-10, and IL-1α in MAFLD patients can be considered a potential diagnostic marker[134,156,157]. In addition, it has been demonstrated that the high level of TNF-α along with the low level of serum IL-10 can be an indicator of the severity of NAFLD in the morbidity of obese men[156], and these two markers have provided promising efficacy in the follow-up of patients with NAFLD[158]. Similarly, it has been suggested that polymorphisms in the gene encoding TNF-α may be a marker of NAFLD progression and risk of coronary artery disease[159]. Accordingly, a 4-year follow-up study has revealed that TNF-α can function as a predictor of NAFLD development[131]. Although TNF-α is one of the triggering factors of the necroptotic cell death pathway and its diagnostic ability can be assumed to be related to necroptosis, further studies to find innate markers of necroptosis appear necessary. Moreover, inflammatory markers are mainly expressed in a wide range of disorders, and the lack of reporting of the sensitivity and specificity of the proposed markers complicates the determination of their diagnostic value.

Pathological alterations in the expression of MLKL, RIPK1, and RIPK3 can be suggested as the most potential markers related to necroptosis in diagnosing NAFLD and predicting its progress[136,150,160,161]. A recent study has determined that metabolomic and lipidomic screening has identified the participation of RCDs, particularly necroptosis, in the progression of NAFLD toward cancer[162], which may be of possible clinical importance in the follow-up of patients and determining the risk of disease progression. Nevertheless, the available evidence is rare, therefore further studies on this content represent a crucial necessity.

Necroptosis as a potential therapeutic target

It was previously discussed that the induction of necroptosis in hepatocytes is related to the occurrence and progression of NAFLD, therefore this pathway of cell death displays the characteristics of a therapeutic target. Several studies have investigated the efficiency of such properties, which can be reviewed in two categories, one contains the direct inhibitors of the mediators of the necroptosis pathway, and the other includes the herbals/chemicals that have revealed therapeutic properties through modulation of necroptosis.

It has been demonstrated that a highly specific inhibitor of RIPK1 known as RIPA-56 was able to ameliorate an animal model of NAFLD *via* down-regulation of MLKL, reduction of hepatic damage, inflammation, fibrosis, characteristic of NASH, as well as of steatosis[163]. In addition, the inhibition of MLKL resulted in a reduction of fat *de novo* synthesis and chemokine ligand expression in patients with NAFLD[164]. Similarly, chemical compounds that inhibited necroptosis along with apoptosis in hepatocytes were able to alleviate NAFLD-related characteristics[165-168]. Due to the cross-talk between RCDs, it is clinically important to note that the process of necroptosis inhibition should not activate other cell death pathways, as the absence of RIPK3 increased

inflammation and hepatocyte apoptosis as well as early fibrotic responses leading to exacerbation of the disease[169].

Interestingly, a variety of herbal compounds such as epigallocatechin gallate, pentoxifylline, kaempferol, quercetin, metformin, *etc.* demonstrated anti-necroptotic properties that benefited the alleviation of NAFLD[170-174]. Regulating lipid metabolism, suppressing destructive inflammatory responses, maintaining cellular homeostasis, and also the rarity of adverse effects are attractive properties that antioxidant compounds provide in the treatment of NAFLD, in addition to inhibiting necroptosis. However, no clinical trials have been registered on the clinicaltrials.gov website, which indicates insufficient current information to confirm treatment strategies based on inhibition of necroptosis. Therefore, the conduct of further studies on this content is pivotally encouraged.

CONCLUSION

The findings of the current review revealed that the induction of necroptosis along with inflammatory responses pivotally contributes to the occurrence of NAFLD. Moreover, the continuation of the necroptotic death of hepatocytes can cause the disease to progress to NASH and HCC. Nevertheless, the diagnostic value of necroptosis-based markers has been rarely evaluated and disease management strategies based on necroptosis necessarily require further investigations in this direction.

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