DISCUSSION

Hepatitis C virus, a member of the *Flaviviridae* family, of the genus *Hepacivirus*, is a hepatotropic, positive-sense, single stranded RNA virus.[7] Persistent infection with HCV is a major risk factor for chronic liver disease worldwide leading to chronic hepatitis and long-term progression to cirrhosis and HCC.[38] During the course of HCV infection, inflammatory milieu, hepatocyte necrosis, steatosis, oxidative stress, and progressive liver fibrosis of variable degrees, eventually result in irreversible cirrhosis.[56] The oncogenic process of HCV infection probably requires multiple steps of genetic and epigenetic alterations and the activation of cellular oncogenes.[67,70] Viral structural as well as NS proteins are accused to orchestrate several hepatocyte signaling pathways with subsequent interference with cellular biological activities.[72] Although the course of HCV infection is influenced by a complex host-virus interplay, yet, the potential mechanism(s) underlying viral persistence and disease progression are not fully understood.[57,60] Intensive research to unravel the multifunctional molecular pathway(s) implicated in the progression of chronic HCV infection, could identify novel therapeutic targets to tackle this devastating disease.[357]

The mTOR is a 289-kDa (2549 AA) evolutionarily conserved serine/threonine protein kinase encoded by *FRAP1* gene. Structurally, the domains found in mTOR, in order from the N to the C terminus of mTOR, compose the so-called HEAT repeats, the FAT domain, the FRB domain, the kinase domain, and the FATC domain.[108] Functionally, mTOR forms two large distinct complexes, called mTORC1 and mTORC2.[114] The mTOR signaling is activated when the genetic and environmental milieu is optimal for cellular growth, and diminishes under stressful conditions including insufficient nutrients, cellular energy depletion, DNA damage and hypoxia.[120,127] The PI3K/AKT pathway is a genuine upstream positive regulator of mTOR.[132,133] The phosphorylation of mTOR promotes downstream targets such as S6K1 and 4E-BP1, positive and negative regulators of protein synthesis respectively.[144] Inhibitors of mTOR such as rapamycin (sirolimus) and rapamycin analogs potently inhibit the dowmstream signaling of mTOR.[147] The mTOR pathway has been shown to function as a central regulator of a wide array of cellular processes [122] such as protein translation,[177,178] cell metabolism,[175] growth, differentiation,[195] and survival [201] and cell cycle progression [196,197] by sensing nutritional status and allowing progression from G1 to S phase in the cell cycle.[196] In addition, mTOR is a key player in the innate and adaptive immune responses.[107] In recent years, increasing evidence demonstrates that mTOR is a down-regulator of the intracellular process of autophagy in response to cellular physiological conditions and environmental stress.[180,287]

Autophagy or 'self-eating' is characterized by the formation of double-membrane vesicles, known as autophagosomes, which sequester cellular constituents and deliver them to the lysosomes. This allows the cell to recycle nutrients and remove unwanted cytosolic components, such as damaged organelles and protein aggregates from the cytoplasm.[257,261] Autophagy involves cascade of events including a series of complexes composed of Atg proteins that assemble autophagosomes.[262] To date, 32 *ATG* genes, that are involved in autophagy, have been identified in mammals.[263,264] Autophagy can be induced by a variety

of stimuli (e.g. nutrient deprivation, hypoxia, cytokines, hormones, viruses and DNA damage).[273] Beyond maintaining cellular homeostasis,[255] autophagy is involved in multiple biological processes including cell quality control, energetic balance, remodeling, and defense against extracellular insults and pathogens.[272]

It is likely that dysregulation of the mTOR-autophagy pathway may contribute to many human disorders including viral infections,[359,360] inflammatory disorders [361,362] and cancer [233,318] and could be an attractive avenue for future therapeutic approaches.[287]

Therefore, the present work was designed to study the role of mTOR and autophagy in the progression of HCV-related liver disease.

mTOR and HCV-related liver disease:

The present study demonstrated that serum mTOR levels were significantly higher in patients with HCV-related liver disease than in healthy subjects and was associated with positive intrahepatic mTOR expression in 60% of patients which was positively correlated with serum mTOR levels. These findings suggest that mTOR was activated during chronic HCV infection. This is the first study to provide data on serum levels and tissue expression of mTOR in patients with HCV-related liver disease. Previous *in vitro* studies showed that mTOR pathway has been activated by HCV infection.[363-368] Using immunoblot analysis, Shrivastava et al (2012) [363] observed an increased expression of total mTOR and phosphorylated-mTOR (p-mTOR) and its downstream substrate 4E-BP1, in HCV-infected Huh 7.5 cells or IHH infected with HCV genotype 2a. Also, Bose et al (2012) [364] demonstrated that both total and p-mTOR (phospho-Ser-2448) and its downstream target phospho-S6K1 were highly expressed in HCV genotype 2a-infected liver cells. Another study found that full-length HCV replicon cell lines increased the activities of PI3K and Akt, as well as the activity of their downstream target, mTOR.[365] Similarly, immuno-precipitation based studies detected an enhanced mTOR activity in human hepatoma cell line (Huh-7) transiently expressing the HCV core protein of genotype 1b.[366] Moreover, George et al (2012) [367] found that HCV NS5A activated mTORC1 and eIF4E in Huh7.5 cells, resulting in enhanced eIF4F assembly. A recent study has also shown that NS5A activated mTOR and subsequently increased the phosphorylation levels of its substrates S6K1 and 4E-BP1 under serum-starved conditions in Huh7 and HCV subgenomic replicon cells. NS5A knockdown abrogated phosphorylation of S6K1 and 4E-BP1 suggesting that NS5A specifically elevated mTOR activity.[368] By contrast, Huang et al (2013) [369] found that the activity of the AKT-TSC-mTOR pathway was inhibited in Huh7 cells either harboring HCV-N (genotype 1b) full-genomic replicon or infected with HCV JFH1 (genotype 2a).

Activation of mTOR signaling pathway may represent one of the various strategies utilized by HCV for the establishment of persistent infection. Both direct and indirect mechanisms might be involved in activation of mTOR by the virus. Many HCV-encoded proteins have been implicated in mTOR activation. The HCV NS5A protein can activate the PI3K-Akt signaling, the upstream of mTOR, by directly binding to the p85 subunit of PI3K.[370] Moreover, the NS5A protein can bind with the cellular protein FKBP38, an

intrinsic antagonist for mTOR activity, thus, disrupting the mTOR-FKBP38 association resulting in mTOR activation.[368] A recent report showed that the HCV NS5A can associate with phosphorylated eIF4E of eIF4F complex through mTOR-4EBP1 pathway and subsequently recruits it to 40S ribosomes and thus, NS5A physically interacts with translation apparatus.[371] Moreover, the HCV core protein from HCV genotype 2a suppressed TSC-1/TSC-2 expression with subsequent mTOR activation.[364] In addition, the core protein of HCV genotype 3a (but not 1b) reduced PTEN protein expression level, a known negative regulator to PI3K-mTOR pathway, in Huh-7 and HepG2 cells leading to up-regulation of mTOR.[372] In the mean time, mTOR activation by HCV proteins may be used as a mechanism which promotes viral replication and orchestrates various cell activities for the virus own benefit leading to HCV persistence and chronicity of infection.[365,368,373-375] It has been shown that activation of mTOR pathway plays a role in viral life cycle. At start of infection, HCV transiently activates the PI3K-AKT-mTOR pathway to enhance its entry into the host cells.[373] In addition, mTOR achieves significant viral protein translation and lipid production needed for virus assembly.[374] It has been shown that stimulation of the mTOR pathway by HCV contributed to the maintenance of steady-state levels of HCV replication [365] while mTOR inhibition by sirolimus treatment or metformin, an activator of AMPK signaling, resulted in a significant decrease in HCV replication.[376,377] Moreover, mTOR proteins protects HCV-infected cells from apoptosis and promotes cell survival contributing to HCV persistence.[365,368] Rapamycin treatment abolished suppression of caspace 3 by HCV NS5A, indicating that NS5A inhibits apoptosis specifically through the mTOR pathway.[368] Also, mTOR controls translation initiation, the major rate-limiting event in eukaryotic protein synthesis, through direct phosphorylation of S6K1 and 4E-BP1. These interactions usually result in upregulation of viral protein synthesis and could be inhibitory to host translation.[367,375] In addition, HCV-induced mTOR can modulate innate and adaptive immune responses by which HCV can subvert the immune response.[107,215,217] mTOR activation negatively controls migration and homing of $CD8^+$T-lymphocytes into secondary lymphoid organs on exposure to pathogens by altering the expression of cell surface receptors.[225] In parallel, mTOR activation also leads to a decrease in the marginal zone of B cells development and a significant reduction in B cell maturation [378] with subsequent delay in the appearance of a protective antibody response to HCV following acute infection, an event that facilitates chronicity of infection.[84]

Many other RNA and DNA viruses have been discovered to activate the mTOR pathway and behave like HCV in gaining benefit from mTOR activation to facilitate viral persistence and pathogenicity.[359] Such viruses include HBV,[379] HIV,[380] West Nile virus,[381] adenovirus,[382] human cytomegalovirus (HCMV),[383,384] herpes simplex virus-1,[385] human *Papillomavirus*,[386] BK polyomavirus [387] and Kaposi's sarcoma-associated herpesvirus.[388] Viruses have evolved mechanisms to keep mTOR complexes active during infection. Some viruses encode proteins capable of activating PI3K-Akt-mTOR signaling in infected cells.[386] Activation of mTOR and its downstream S6K1 by HBV X gene seems to depend on IKKβ.[379] Likewise, herpes simplex virus-1 activates mTOR through virus-encoded Us3 Ser/Thr protein kinase which acts analogously to the cellular kinase Akt.[385] Moreover, HCMV activates mTOR via an interaction between the viral UL38 protein and the TSC, which inactivates the TSC, the negative regulator of mTOR.[389] In addition, the virus maintains mTOR activity during stress responses by inducing the expression of

antioxidant and detoxifying enzymes to protect mTOR from inhibition by oxidative stress, which is critical for the success of the viral infection.[390] Also, HCMV maintains mTOR activity via sequestration of mTOR and its activator Rheb-GTP, in the viral assembly compartment to protect mTOR from inhibition by cellular stress responses induced during lytic infection and protein deprivation.[383]

mTOR and HCV-related hepatic inflammation, fibrosis and steatosis:

In addition to its role during viral infections, mTOR has a crucial regulatory effect on chronic inflammation, fibrosis and steatosis and may play a role in the pathogenesis of HCV-related liver damage.[391] In the present study, the serum mTOR levels and intrahepatic mTOR staining score were positively correlated with serum levels of aminotransferases and the METAVIR histological activity grade in patients with HCV-related liver disease suggesting that activation of mTOR pathway was associated with an inflammatory environment in HCV-infected livers. Activation of mTOR has been also reported in other experimental inflammatory liver diseases such as NASH [392] and immune-mediated hepatitis.[223,393] Wang et al (2014) [392] demonstrated that hepatic mTOR, phosphorylated mTOR (Ser2448) and S6K1 expression tended to increase significantly with the progression in pathological morphology from simple fatty liver to fatty hepatitis (NASH) in the high-fat fed mice, with a significant increase in the inflammatory profiles of the liver which was decreased by rapamycin treatment. Moreover, a significant positive correlation has been found between p-mTOR(Ser2448) expression and the proinflammatory cytokine TNF-α mRNA in the livers.[392] Induction of hepatic inflammatory stress by casein injection in C57BL/6J mice in vivo or by TNF-α or IL-6 treatment of human hepatoblastoma HepG2 cells, was associated with activation of mTORC1 and upregulation of phosphorylation of its primary substrates, S6K and 4E-BP1.[394] In addition, mTOR inhibition decreased inflammatory gene expression in the livers of mice deficient in Raptor, the regulatory associated protein of mTOR in an immune cell-mediated hepatitis model.[223] In bile duct ligation (BDL) rats, treatment with rapamycin improved liver function greatly and distinctly decreased ALT, AST, alkaline phosphatase, total bilirubin, and bile acids paralleled by an reduction of the amount of intrahepatic neutrophils and lymphocytes as well as hepatocellular injury.[395] Menon et al (2012) [396] observed an increased serum concentrations of ALT and AST, focal areas of necrosis and inflammation with macrophage infiltration in genetic mouse model with liver-specific knockout of TSC1, a negative regulator of mTOR, while rapamycin treatment blocked liver damage in these mice. Also, a recent study found that the mTOR inhibitor everolimus was more effective than calcineurin inhibitors (CNI) in reducing hepatic macrophage infiltration and expression of TNF-α and inducible NO synthase (iNOS) mRNA in BDL rats suggesting a superior impact of mTOR on inflammatory processes.[397]

The relationship between mTOR signaling and the inflammatory response could be possibly mutual. It has been shown that inflammatory cytokines such as TNF-α may activate mTOR through the PI3K-Akt pathway.[140,141] Moreover, TNF-α activates IKKβ which physically interacts with and inactivates TSC1, leading to mTOR activation.[141] Also, the phosphatedic acid liberated from macrophages during the inflammatory reaction was found to increase mTOR signaling.[398] In the mean time, mTOR activation may play a

central role in the inflammatory response. mTOR promotes the expression of proinflammatory cytokines like TNF-α, IL-6, IL-1α and IL-1β.[392,395,397] mTOR phosphorylation is necessary for the activation of NF-κB, which induces high expression of TNF-α suggesting a complex interaction between TNF-α, NF-κB and mTOR.[399] Also, mTOR kinase regulates the rate of iNOS mRNA stability to exert its proinflammatory effects via NO synthesis and rapamycin was found to increase in the rate of iNOS mRNA degradation with subsequent amelioration of inflammatory events.[400] In addition, mTOR activation enhances the expression of monocyte chemotactic protein-l and hepatic macrophage infiltration and also modulates macrophage polarization with M2 to M1 phenotype switch promoting proinflammatory cytokine production and tissue inflammation [218,401] and is essential in TLR2- and TLR4-induced neutrophil activation.[220,221] Also, mTOR pathway promotes the IL-6-induced IL-17 production by $CD4^+$ T cells and enhances the proinflammatory Th17 response through STAT3 activation.[402] Likewise, the mTOR pathway positively regulates invariant NKT cell functions upon stimulation with a cognate antigen and is critical for the development of invariant NKT (iNKT) cell-mediated hepatitis.[223] Meanwhile, mTOR antagonizes differentiation of naïve $CD4^+$ cell to $Foxp3^+$ Treg cells which suppress immune-cell proliferation [231,403] and it also limits the recruitment of myeloid-derived suppressor cells to inflammation sites which are critically required for protection against hepatic injury.[393,404]

Furthermore, accumulating evidence has suggested a potential role of mTOR pathway in hepatic fibrogenesis.[404-409] In the present study, serum mTOR levels and intrahepatic mTOR expression score were positively correlated with the METAVIR fibrosis stage patients with chronic HCV infection and there was a progressive increase in serum mTOR levels from CHC to HCV-related cirrhosis. Previous studies observed that the use of sirolimus, an mTOR inhibitor, was associated with a significant decline in the rate of liver fibrosis in post-liver transplant patients.[404-406] Also, liver transplant recipients on everolimus monotherapy had lower serum expression of the fibrosis markers hylorunic acid, an essential component of ECM, and transforming growth factor beta (TGF-β), the most potent stimulus for hepatic fibrogenesis, than patients on CNI.[407] The potential role of mTOR pathway in the fibrogenic process has also been demonstrated in experimental models of liver fibrosis. Wang et al (2014) [395] found that mTOR expression in the liver assessed by western blot was markedly activated in the early phase of cirrhotic portal hypertension induced by BDL in rats and that mTOR inhibition reduced the amount of cholangiocyte proliferation and intrahepatic fibroblasts and ECM deposition as well as numbers of periductular activated hepatic stellate cells (HSCs). Similarly, a recent study found that mTOR blockage with everolimus and to a lesser extent sirolimus, decreased hepatic fibrosis up to 70%, significantly reduced numbers of cholangiocytes and myofibroblasts, and lowered hepatic hydroxyproline and procollagen-α1 mRNA in an experimental liver fibrosis model.[397] After BDL- and thioacetamide-induced cirrhosis, low dose rapamycin reduced fibrogenesis and the accumulation of ECM-producing cells and ECM components than in vehicle-treated cirrhotic rats.[408] Also, the natural product Leukamenin F ameliorated the progression of carbon tetrachloride-induced liver fibrosis in mice by inhibiting the phosphorylation of mTOR and its downstream target p70S6K leading to reduced HSC proliferation and collagen gene expression.[409] In addition, several investigators demonstrated that the inhibition of hepatic fibrosis after mTOR pathway

blockade was associated with improvement of portal hypertension in established cirrhotic animal models as indicated by a significant decrease in portal pressure,[395,397,408,410] amelioration of splenomegaly [395,408,411] and reduction in the development of ascites.[397] Moreover, Mejias et al (2010) [411] demonstrated that the activity of mTOR signaling was overactivated in the spleen of portal hypertensive rats, and that chronic mTOR blockade by rapamycin led to a dramatic regression of splenomegaly, an effect that was most likely due to the inhibitory action of rapamycin on cellular proliferation, angiogenesis and fibrogenesis.

The mTOR signaling pathway would have multiple mechanisms of action to promote hepatic fibrosis. Being a central mediator of inflammation, mTOR could aggravates the fibrogenesis process by enhancing immune-mediated inflammatory responses in the liver.[219,395,397] Moreover, the mTOR signaling pathway plays a central role in key steps of hepatic fibrogenesis. It has been shown that mTOR promotes the proliferation and activation of HSCs, the key fibrogenic cells in the liver. [395,397,412,413] mTOR signaling stimulates phosphorylation of p70S6K, which regulates protein synthesis and enhances DNA synthesis and cyclin D1 expression leading to HSC growth and proliferation.[413] Also, mTOR enhances transdifferentiation of quiescent HSCs to activated myofibroblast-like cells and up-regulation of α-smooth muscle actin (α-SMA) expression, as a marker of activated HSCs,[413,414] In addition, Akt/mTOR activation was found to be important for both contraction and migration of HSCs to areas of liver tissue damage as part of a coordinated effort aimed at tissue repair.[414,415] Furthermore, mTOR promotes collagen formation in the liver with procollagen-α1 and type I collagen expression [395,397,413,416] and inhibits matrix degradation by matrix metalloproteinase (MMP)-3 and MMP-13 in activated HSCs.[397] The mTOR pathway is involved in the regulation of collagen production through enhancing posttranslational procollagen to collagen biosynthesis pathways (PLOD, PCOLCE, and P4HA) in normal and pathologic fibroblasts. PLOD encodes for an enzyme that hydroxylates lysyl residues within collagen peptides, producing irreversibly cross-linked collagen fibers that are characteristic of scar accumulation.[417] Also, recent studies linked mTOR to the signaling pathway of the $\alpha_V\beta_3$integrin, a receptor for vitronectin, which is the primary molecule required for fibroblast attachment and spreading.[418] In addition, mTOR/p70S6K enhances fibrogenesis through direct effect on the expression of pro-fibrotic genes like TGF-β1, PDGF, PDGF-receptor beta, tissue inhibitor of metalloproteinase 1, fibulin and plasminogen activator inhibitor-1 in activated HSCs and portal fibroblasts.[395,397,401,419,420] Thus, retardation of liver fibrosis by mTOR inhibition seems to be related primarily to blockage of HSC proliferation and activation, downregulation of fibrogenic genes, and upregulation of fibrolytic enzymes.[397,401,410,415,421]

Another possible mechanism that promotes liver fibrosis progression is the development of hepatic steatosis, which is a common histological feature of chronic HCV infection.[51] Hepatic inflammatory stress may exacerbate lipid mediated hepatocyte injury.[394] The present study showed that serum mTOR levels and intrahepatic mTOR expression were positively correlated with degree of hepatic steatosis in patients with HCV-related liver disease suggesting a possible role of mTOR in lipid accumulation in the liver. Using cDNA microarray, Kubrusly et al (2010) [422] found that the mTOR gene and

protein expression was up-regulated in liver samples from patients with NASH/ NASH-related cirrhosis in comparison to normal livers and cirrhosis of other etiologies. Another recent study, also, demonstrated activation of mTORC1 signaling with hepatic lipid accumulation in liver samples from patients with NAFLD and NASH and an elevation of genes downstream of TORC1 signaling and lipogenic genes in NAFLD models while rapamcyin treatment prevented the development of hepatic steatosis.[423] Similarly, Wang et al (2014) [394,424] showed that rapamycin inhibited the phosphorylation of mTOR and its downstream translational regulators p70S6K, 4E-BP1, and eIF4E and reduced hepatic uptake of free fatty acids and triglycerides with the alleviation of hepatic steatosis induced by inflammatory stress in human hepatoblastoma HepG2 cells. Moreover, mRNA and protein levels of mTOR and S6K1 were significantly increased in association with a significant decrease in the phosphorylated insulin receptor substrate-1 (IRS-1) level in the high fat-fed rats when compared with those in rats on a standard diet contributing to the development of hepatic steatosis and IR.[392]

It has been established that HCV infection promotes hepatic steatosis and IR and has been associated with metabolic syndrome and fibrosis progression.[425] Moreover, HCV-related steatogenesis is required for stable viral replication. In infected cells, lipid droplets serve as intracellular storage organelles for HCV core protein and are essential for virus assembly.[426] HCV affects lipid metabolism by several molecular mechanisms, which could be mediated through mTOR activation.[168,426] mTOR is known to regulate both anabolic and catabolic lipid metabolism by regulating lipogenesis and lipolysis respectively at the mRNA expression level.[186] At the molecular level, mTOR was found to regulate lipogenesis through negative regulation of lipin-1 and/or activation of S6K1 with subsequent activation of SREBP-1, the key transcription factor that activates genes involved in lipid and cholesterol metabolism such as fatty acid synthase through binding of the sterol regulatory element in the gene promoters.[188,190] Also, mTOR activates stearoyl CoA desaturase, a key enzyme in fatty acid metabolism required for double bond formation.[189] Moreover, mTOR signaling upregulates PPARγ through inhibition of 4E-BP1 and/or inhibition of lipin-1. The PPARγ induces the expression of genes which promote fatty acid uptake, synthesis, esterification, and storage. Meanwhile, mTOR inhibits triacylglycerol lipolysis suggesting the importance of mTOR in blocking catabolic pathways.[186,187] In addition, activation of the mTOR signaling pathway increases hepatic CD36 translational efficiency and CD36 protein expression, which facilitates fatty acid uptake and triglyceride storage resulting in liver fat accumulation, hyperinsulinaemia and IR.[394,424,427] Also, activation of the mTOR/S6K1 cascade may prompt the development of IR through interference of intracellular insulin signaling, mainly, the serine phosphorylation of IRS-1, which acts as a homeostatic negative feedback loop between nutrient- and growth factor-dependent signaling networks.[392] Inhibition of mTOR alleviates hepatic steatosis by upregulating the expression of genes that promote fatty acid oxidation and downregulating genes that participate in fatty acid synthesis and protects against IR.[428] Metformin, the most commonly used oral antidiabetic agent, is considered to suppress mTOR/S6K1-mediated IRS-1 phosphorylation in an AMPK-dependent fashion, and thus enhances insulin sensitivity in the liver and, to a lesser extent, in the muscle.[429] The fact that HCV core protein is able to induce gene expression and activity of SREBP1,

PPARγ and CD36 may suggest that HCV-induced mTOR activation could be one of the mechanisms by which the virus promotes hepatic steatosis and IR.[425,427]

mTOR and HCV-related hepatocellular carcinoma:

As a key regulator of cell growth and proliferation,[136,176] mTOR has been the subject of intense investigation for its role in tumor development and progression.[166,173,233] Upregulation of the mTOR pathway has been seen in several human solid cancers.[430-432] The present study demonstrated that development of HCC in patients with HCV-related liver disease was associated with a further significant increase in serum mTOR levels and intratumoral mTOR staining intensity which was significantly higher than the surrounding non-neoplastic liver tissues and was positively correlated with serum AFP levels and tumor size, Edmonson grade and CLIP stage. These findings indicate that mTOR activation progressed from chronic hepatitis to cirrhosis to HCC in HCV-related liver disease and was associated with aggressive tumor behavior. Several lines of evidence implicate the mTOR pathway in hepatocarcinogenesis with upregulation of mTOR and many components of its upstream signaling (PI3K, PTEN and AKT) and its downstream effectors (S6K1, 4EBP1 and eIF4E) in cancerous tissues.[234-236,433-445] Kang et al (2014) [433] demonstrated positive immunohistochemical expression of mTOR in 80/83 HCC patients (96.4%); mTOR high expression (2+, 3+) was observed in 51/83 HCCs and low expression (negative, 1+) was detected in 32/83 HCCs.[433] In another study, p-mTOR expression evaluated by immunohistochemistry on tissue microarrays was detected in the cytoplasm of the malignant cells in 47.5% of HCCs while all adjacent non-cancerous liver tissues were negative for p-mTOR staining.[434] Moreover, Hui et al (2009) [435] found that 67% of human HCCs showed at least a two-fold increase in the mTOR mRNA expression level using real-time quantitative reverse transcriptase PCR when compared with their corresponding non-tumorous livers. In addition, the intensity of immunostaining of the phosphorylated forms of the three key constituent proteins of the Akt-mTOR-p70S6K signal pathway were significantly increased in HCC with different etiologies including HCV infection compared with hepatocellular adenoma, cirrhotic nodules and normal liver tissues.[436] Similarly, Calvisi et al (2011) [437] found a gradual induction of activated AKT, mTOR and RpS6 in HCC and non-neoplastic surrounding livers when compared with normal livers, with the highest expression being observed in HCC. Also, the expression frequencies of the mTOR upstream AKT and its effector pS6 were found to be generally increased while expression of PTEN, the negative regulator of mTOR, was decreased in HCC tissues compared with adjacent non-tumor and normal liver tissues.[438] Moreover, activation of the Akt-mTOR pathway in HCC was evidenced by an incremental increase pS6 immunostaining in HCC, followed by dysplasia, surrounding cirrhotic tissue and cirrhotic tissue in patients without HCC and by loss of PTEN expression which was more common in patients with higher tumor stage, compared to those with stage 1 tumors.[439] In a large human HCC tissue sample cohort, mTOR signaling (phosphorylated- RpS6) was aberrantly up-regulated in 47.7% of HCCs as determined by integrating data from direct sequencing, DNA copy number changes and mRNA levels and in 67.3% of HCC using immunohistochemistry and was significantly higher than surrounding cirrhotic tissue.[440] Meanwhile, activation of the mTOR signaling has been associated with poor prognosis of HCC where aggressive characteristics, such as poor differentiation, high TNM and BCLC

staging, higher levels of AFP, frequent intrahepatic metastasis, vascular invasion, angiogenesis and high proliferation index of HCC were significantly more frequent in patients with high expression of the mTOR pathway components.[433,434,436,438,440] Moreover, a high level (>75% positive cells) of phosphorylated-RPS6 immunostaining in HCC tissue from liver explants was associated with more rapid post-transplant HCC recurrence after orthotopic liver transplantation.[441] Inhibition of mTOR can suppress tumor growth and sensitize tumor cell to chemotherapy or other target therapy.[442,443] Besides the prognostic potential of mTOR, the present study showed that serum mTOR levels could be of diagnostic value for the development of HCC in patients with HCV-related cirrhosis with a sensitivity and specificity of 92.2% and 100% respectively at a cut-off value of 4.55 ng/ml; a finding that should be verified in a large number of patients

Furthermore, the biologic rationale for the role of mTOR in the pathogenesis of HCC has been supported by several preclinical studies. Menon et al (2012) [396] found that liver-specific TSC1 knockout mice showed constitutively elevated mTORC1 signaling and developed sporadic HCC, which was preceded by sequential development of histological features associated with HCC including liver damage, inflammation, necrosis, and regeneration. Moreover, the mRNA and protein levels of mTOR and its downstream targets p70S6K, S6, and 4EBP1 were overexpressed in HCC cell lines compared with normal cells using semi-quantitative RT-PCR and Western blotting.[435,444,445] In addition, blockage of mTOR showed anti-neoplastic activity in experimental models of HCC. Human liver cancer cells treated with the TOR inhibitors sirolimus, everolimus and temsirolimus showed G1 phase arrest and decreased cell viability and proliferation.[435,440,444,445] In an experimental HCC xenograft model, everolimus treatment decelerated tumor growth at 15 days compared with placebo and significantly expanded median overall survival.[440] Moreover, mTOR blocking by siRNA interference reduced the migration and invasion abilities of liver cancer cells.[445]

The mTOR signaling pathway represents a central avenue for several oncogenic pathways. Aberrant activation of mTOR in the majority of human cancers often occurs as a result of dysregulation of a signaling network of oncogenes and tumor suppressors lying upstream of mTOR.[237] The mTOR pathway integrates signals from pro-oncogenic growth factors such as IGF-1, epidermal growth factor and VEGF [136] as well as from inflammatory cytokines such as TNF-α/IKKb signaling pathway.[141] Cellular energy levels, cellular stress, hypoxia and DNA damage also modulate the mTOR pathway.[127-130] These upstream signals activate PI3K, which in turn up-regulates the protein kinase Akt and subsequently mTOR signaling.[132,133,436,437] Moreover, PTEN, a tumor suppressor protein that negatively regulates the mTOR pathway, is inactivated in approximately half of HCCs through deletion, silencing or mutation, leading to up-regulation of mTOR-dependent pathways.[134,434,438,439] In addition, mutation or loss of heterozygosity in TSC, an upstream negative regulators of mTOR, leads to cancers through loss of its inhibitory effect on mTOR.[396] Also, the loss of p53, a very common event in cancer, promotes mTOR activation.[237]

In the last few years, significant advances have been made in understanding the role of mTOR in cancer development and progression.[173] The participation of mTOR as a

survival pathway in tumerogenesis can be explained by its direct effect on the cell metabolism and biology and indirect effect through working as common modulator of other oncogenic signaling pathways.[233] Being a key regulator of cellular metabolism, the mTOR pathway links oncogenic signaling with metabolic changes, which contribute to the bioenergetic and anabolic demands of rapidly growing and proliferating tumor cells.[173,176] Activation of mTOR accelerates cell growth through promoting protein synthesis where mTOR mediates activation of ribosomal S6K, which in turn enhances the translation of mRNAs required for protein synthesis.[148,149] Also, phosphorylation and inactivation of 4E-BP1 suppressor proteins by mTOR activation leads to the formation of the eIF4F complex which facilitates translation of various malignancy-associated mRNAs that encode pro-oncogenic proteins such as cell cycle regulators (cyclin D1), ornithine decarboxylase, proto-oncogenes (*c-myc* and VEGF) and apoptosis inhibitors (Bcl-2, Bcl-xL and Mcl-1).[150,151,238,446] In addition, the activation of the PI3K-Akt-mTORC1 pathway, have been found to promote glucose uptake and its glycolytic conversion to lactate, known as aerobic glycolysis, which provides tumor cells a proliferative advantage over normal cells under conditions of intermittent hypoxia.[447] Moreover, mTOR pathway promotes lipogenesis via activation of PPARγ and SREBP-1,[186-188] which enhance proliferation and survival of HCC cells.[188,437] Aberrant lipogenesis with liver fat accumulation seen in non-tumorous liver tissue in association with mTOR activation, may be one of the mechanism through which HCC develops on the basis of NASH and chronic HCV infection.[448,449] Beside its metabolic roles, the mTOR pathway promotes cell proliferation and cell survival by inhibiting apoptosis and cell cycle arrest with subsequent progression from the G1 to the S phase of the cell cycle leading to accumulation of mutations and tumor development.[195] Moreover, activation of mTOR in tumor cells enhances tumor angiogenesis by activating VEGF signaling pathway and the translation and stabilization of HIF-1 as well as the expression of other angiogenic factors such as NO and angiopoietins during hypoxia.[156,182,239,436] Tumors that form as a result of mTOR activation are highly vascularized [436] and inhibition of mTOR significantly reduces tumor growth primarily via its anti-angiogenic potential.[450] Furthermore, the activation of mTOR can confer many growth advantages to cancer stem cells such as promoting proliferation, survival, maintenance of pluripotency and resistance to apoptosis induced by various stress signals such as hypoxia and nutrient deficiency.[451] In addition, mTOR can regulate telomerase activity at post-transcriptional level in hepatocarcinogenesis by up-regulating human telomerase reverse transcriptase (hTERT) protein level.[452] Recently, the association between mTOR expression and poor tumor criteria was found to be related in part to the ability of mTOR to mislocalize the p27 into the cytoplasm rather than nuclear localization, which is required for its tumor suppressor functions.[453] Moreover, association of mTOR activation with the intrahepatic and vascular metastasis of HCC have been linked to high expression of the zinc-dependent endopeptidases MMP-9, which facilitate capsular invasion via degradation of ECM and the basement membrane.[434]

Autophagy in HCV-related liver disease:

Autophagy is the basic catabolic process by which cells degrade unnecessary or dysfunctional cellular components through the lysosomal machinery.[256,288] In the liver, autophagy plays important roles, including the balance of nutrients and energy for basic

cell functions, the removal of misfolded proteins, and the turnover of major subcellular organelles such as mitochondria, ER, and peroxisomes under both normal and pathophysiological conditions. Therefore, disturbances in autophagy function in the liver might have critical effects on liver physiology and liver disease.[454] The present study showed that Atg5, an autophagy marker, was detectable with variable staining intensities in hepatocytes of most patients with HCV-related liver disease. Among the Atg proteins, Atg5 is a key element of autophagosome elongation and is essential in proceeding of autophagy. Conjugation of Atg5 with Atg12 interacts with Atg16 to form a multimer complex, which is localized to membranes of early autophagosomes.[269] There are limited data concerning the assessment of autophgy in patients with chronic HCV infection.[455-457] Using immunoblotting, Rautou el (2011) [455] showed that the level of LC3-II protein, a main regulator of autophagy, was significantly higher in the liver of CHC patients than in controls with other liver diseases, namely, chronic HBV infection, NASH or alcoholic liver disease as well as patients with no or mild liver abnormalities. This was supported by the finding of a sixfold increase in the number of autophagic vesicles using electron microscopy in hepatocytes from CHC patients compared with overall controls unrelated to viral load or HCV genotype.[455] In another recent study, Beclin 1 mRNA expression was constitutive of all liver specimens from patients with HCV-related and HBV-related chronic hepatitis and cirrhosis and the highest Beclin 1 mRNA levels were found in cirrhotic liver tissues with no correlation with the HCV genotype.[456] Moreover, several *in vitro* studies have suggested that the autophagic pathway was activated in HCV RNA-expressing cultured cells.[458-462] Ait-Goughoulte's group demonstrated that autophagic vesicles and GFP-LC3-labeled punctate structures accumulated in immortalized human hepatocytes (IHH) transfected by the HCV genotype 1a genome and was accompanied by upregulations of Beclin and the Atg12-Atg5 conjugate, the major characteristics of autophagic process.[458] Also, the HCV-induced autophagosome formation was detected in the Huh7 cells transfected with HCV genotype 2a JFH1 viral RNA.[459] Similarly, Dreux et al (2009) [460] showed that the content of autophagy vesicles as well as the autophagy initiation proteins Beclin-1, Atg12 and Atg5 were increased in JFH1 HCV-infected cells. Also, it has been found that the autophagy vacuoles were increased at the subcellular level within peri-nuclear regions of infected cells as well as Atg5 in HCV infected cells.[461]

The molecular mechanism underlying how HCV activates autophagy has been poorly understood so far. Multiple interactions between HCV and autophagy proteins have been identified and resulted in autophagy induction. By yeast two-hybrid assays, NS5B, NS5A, and p7 were found to interact with Atg5, Atg12, and FIP200 respectively.[461,462] Moreover, immunoprecipitation assays revealed the ability of NS4B to copurify with the Beclin 1-associated lipid kinase Vps34, as well as p7 with Beclin 1 as a mechanism for HCV-induced autophagosome formation.[463,464] The core protein has been shown to activate both ATF6 and PERK pathways that, in turn, increase the expression of Atg12 and LC3 genes.[465] Moreover, it has been proposed that HCV induces autophagy through ER stress and the unfolded protein response (UPR) pathway in hepatocytes which are mediated by the HCV NS4B and NS2 proteins.[459,466] The release of calcium from ER to cytosol is known to impair mitochondrial activity and creates oxidative stress, which are potent autophagy inducers.[467] In addition, HCV could control autophagy by modulating the activity of the cytosolic RNA-sensing PKR, which has been reported to regulate virus- and

starvation-induced autophagy.[468] Also, the HCV NS3 could modulate autophagy *via* the interaction of the immunity-associated GTPase family M (IRGM) with Atg5 and LC3.[462]

However, whether HCV induces a functional or incomplete autophagy process remains controversial. Emerging lines of evidence suggested that HCV stimulates an incomplete autophagic response. Sir et al (2008)[459] showed that HCV induced autophagosome formation but was not able to enhance autophagic degradation of long-lived proteins and p62 in JFH1 RNA-transfected cells suggesting an inefficient fusion between autophagosomes and lysosomes. In patients with chronic HCV infection, an electron microscopy analytic study found an increased number of early autophagic vesicles in hepatocytes from CHC patients but did not find late autolysosomes or an increase in the number of mature lysosomes with electron-dense contents that could attest such a blockade of the last step of autophagy.[455] A previous study has shown that ectopic expression of HCV NS4B was sufficient to activate incomplete autophagy by interacting with Rab5 and Vps34 in human hepatoma cells.[463] It has been suggested that HCV may utilize Atg5 to initiate translation and double membrane vesicle formation leading to relative non bioavailability of Atg5 with no further autophagosome formation and subsequent incomplete autophagy activation.[461] Moreover, it has been proposed that the ability of HCV to inhibit the autophagic flux may depend on the viral genotype. Cells harboring an HCV replicon RNA of genotype 1b strain Con1 exhibited an incomplete acidification of the autolysosome due to a lysosomal defect while those with the genotype 2a strain JFH1 have no alterations.[469] Indeed, mature lysosomes are the final product of the autophagic process.[264] The mechanism of the blockade of mature lysosome formation has not yet been clarified; however, a decrease in the lysosome-associated membrane protein (LAMP) 2 was suggested as a mechanism.[470] In addition, modulation of the cellular redox state by catalase expression is able to reduce autophagy levels in HCV replicon cells.[467] On the other hand, previous studies showed that HCV-induced autophagosomes are capable of maturing, as demonstrated by the presence of both the initial- and late stage autophagic vacuoles and by the accumulation of HCV-induced LC3-II by interfering with autolysosome degradation.[460,466] In this regards, it should be noted that the incomplete autophagy induced by HCV may represent a strategy for the virus to escape lysosomal degradation; a process termed "xenophagy".[459] Moreover, HCV may disrupt the complete autophagy process to overcome the autophagy-mediated degradation of intracellular lipid droplets (lipophagy) and this may explain the excess accumulation of lipid and steatosis in HCV-infected cells.[457] The HCV life cycle is tightly coupled to the lipid metabolism of host cells. The HCV virions are bound to lipoproteins, called "lipoviroparticles," and the ER-associated lipid droplets are the viral assembly sites where the nucleocapsid protein core and the replication complex interact to initiate capsid assembly.[426]

Interestingly, it has been demonstrated HCV not only induces autophgy but also use components of the autophagy machinery for its own benefit.[471] The membranous structure of viral-induced autophagosome may be utilized as the replication site of HCV.[472] Also, autophagy contributes directly or indirectly to the cytoplasmic transport of the incoming HCV RNA to cellular factors or sites that are required for virus translation.[460] Moreover, HCV exploits autophagy to escape innate antiviral immunity, thus, promoting viral infection. Autophagy inhibits the retinoic acid inducible gene I protein recognizing

sequences in HCV RNA genome [473] and thus, blocks the induction of IFN-α and β and IFN-stimutated genes.[363] In addition, HCV induces a selective type of autophagy called "mitophagy" that removes damaged mitochondria to sustain the survival of HCV-infected cells.[303,474] However, HCV-induced autophagy is required by the virus only early in the infection for initiation of its replication.[460] Once replication is established, autophagy proteins are not needed for HCV life-cycle since down-regulation of autophagy proteins 10 days after transduction had no effect on maintenance of HCV replication or viral particle release.[475] Thus, HCV might usurp the autophagy pathway to use it as a mechanism of initiating HCV RNA replication and thereafter, it might inhibit autolysosome maturation to protect itself from xenophagic degradation.[459,472]

Autophagy and HCV-related hepatic inflammation and fibrosis:

Since autophagy serves as an essential cytoprotective response to pathologic stresses, dysfunction of autophagy in chronic HCV infection may result in liver injury. The present study demonstrated that intrahepatic Atg5 staining score was inversely correlated with serum levels of aminotransferases and the METAVIR histological activity grade and fibrosis stage in patients with HCV-related liver disease. A previous study showed that interference with autophagy in Con1-HCV (genotype 1b)-transfected cells triggered severe cytoplasmic vacuolation and cell death.[459] Also, deletion of Atg7, an essential autophagy gene, in the mouse liver leads to severe liver injury with hepatomegaly and accumulated excessive numbers of peroxisomes, deformed mitochondria, and concentric membranous structures, in addition to ubiquitin-positive aggregates in hepatocytes, which could be anticipated to cause liver dysfunction in the long run.[476] Moreover, induction of autophagy almost completely suppressed acetaminophen-induced injury in primary cultured hepatocytes and in mouse livers.[477] In addition, inhibition of autophagy made hepatocytes more susceptible to cell death caused by menadione- and TNF-induced hepatic injury *via* the mitochondrial death pathway [478,479] and worsened liver ischemia/reperfusion injury in the late phase.[480] Meanwhile, animal models of ethanol-induced steatohepatitis and NAFLD exhibited decreased hepatic autophagy and stimulation of autophagy reduced liver cell injury.[481,482] Also, patients with proven NAFLD demonstrated decreased LC3 and increased p62 immunostaining with an increased degree of steatosis in their liver biopsies, suggesting decreased autophagy in more severe steatosis.[483] Although autophagy acts primarily as a pro-survival signal, it could also, under certain conditions, lead to autophagic cell death, resulting in excessive catabolism, misrecognition of cargo, and/or hijack of the apoptosis machinery.[288,307] For example, overexpressed Atg5 could promote apoptosis via its interaction with FADD104 or Bcl-xL.[484] Thus, autophagy may prevent death under milder conditions while excess activation of autophagy may represent a failed adaptive mechanism and may contribute to cell death through unchecked degradative processes.[308] The complicated relationship of autophagy and cell death has not been well understood in liver diseases.[309,454]

Autophagy has emerged as a key mechanism in inflammation resolution through suppression of inflammasome activation.[485] Under sterile conditions, autophagy clears the cytoplasm of debris, protein aggregates and defective organelles that can function as endogenous inflammasome agonists.[486] Activation of autophagy is essential for rapid

removal of apoptotic corpses that are crucial for prevention of unwanted inflammation.[487] Also, autophagy protects cells from oxidative stress through removal of damaged mitochondria via mitophagy.[478] Cells with defective autophagy leads to an accumulation of dysfunctional mitochondria that leak endogenous inflammasome agonists, such as mitochondrial DNA and ROS with subsequent augmentation of inflammation.[488] Moreover, autophagy suppresses the secretion of the proinflammatory cytokines such as TNF-α, IL-1β, IL-17 and IL-18 specifically in response to TLR4 activation.[489] Accumulation of damaged mitochondria in autophagy-deficient macrophages and release of mitochondrial DNA into the cytosol, leads to increased production of the inflammatory cytokines through the NALP3 (a NOD-like receptor family member) inflammasome.[490] In addition, derangement of autophagy leads to impaired degradation and accumulation of p62, an autophagy substrate, that results in NF-κB activation and a subsequent increased production of pro-inflammatory cytokines and cell injury.[491]

Nevertheless, the role of autophagy in the process of liver fibrosis is controversial. Autophagy may promote liver fibrosis by enhancing HSC activation with loss of intracellular lipid droplets via lipophagy and transition into myofibroblast.[492] Since HSC activation is an energy consuming process, lipophagy in HSC cells may provide a key energy source of free fatty acids from the breakdown of lipid droplets to fuel HSC activation.[493] It is therefore possible that selective inhibition of autophagy in liver fibrogenic cells might be used to treat patients with liver fibrosis. However, since the fibrogenic cells only account for a small portion of the cells in the liver, it is not clear how the drug would specifically target fibrogenic cells without affecting other cell types.[492,493] On the other hand, data also exist that support an anti-fibrosis role of autophagy. Increased collagen deposition and fibrosis in the kidney was observed in Beclin 1 heterozygous deletion mice, suggesting autophagy may suppress fibrosis. Interestingly, collagen is found to be co-localized with LC3 positive vesicles and in LAMP1 positive lysosomes, suggesting that collagen might be degraded via the autophagy pathway.[494] Since autophagy plays a hepatoprotective role, liver fibrosis might be triggered by the chronic inflammatory response, stresses (ER and oxidative) and steatosis induced in autophagy-deficient cells, which are more vulnerable to injury.[478,481,485] Therefore, further studies are needed to clarify the role of autophagy in the process of liver fibrosis before a therapeutic approach targeting autophagy can be used to treat patients with liver fibrosis.

Autophagy and HCV-related hepatocellular carcinoma:

Autophagy is believed to be important in tumorigenesis and tumor progression.[318,333] Using immunohistochemical staining, the present study demonstrated that the staining intensity of Atg5 was significantly lower in HCV-related HCCs than in chronic HCV infection tissues without HCC and the surrounding non-neoplastic liver tissues and was inversely correlated with serum AFP levels and tumor size, histological grade and stage while there was no statistically significant difference between the chronic HCV infection tissues and the surrounding non-neoplastic liver tissues. These findings suggest that autophagy is a tumor suppressor mechanism and a downregulated autophagy may contribute to the development and progression of HCC. Using real-time PCR and western blotting, Kotsafti et al (2012) [495] showed that the mRNA levels of Beclin 1, an

important autophagic gene, were lower in human HCC tissues than in chronic hepatitis or cirrhosis in the absence of HCC (HCV- and HBV-related) and the highest levels were found in cirrhotic liver tissues while there was no difference with the corresponding non-cancerous tissues. The Beclin-1 mRNA levels were correlated with recurrent disease and free-disease survival suggesting that autophagy was correlated with a good prognosis.[496] Similarly, Qiu et al (2014) [496] found that the Beclin-1 expression detected immunohistochemically in tissue microarrays was significantly lower in HCC tissues than adjacent nontumor liver tissues and was negatively correlated with cirrhosis background, Edmondson grade, vascular invasion, proliferation, and angiogenesis and positively related with overall survival rates. Also, the expression of autophagy-related markers, LC3 and Beclin-1, was positive in 21.1% and 5.8% of the HCC specimens respectively by immunohistochemistry and LC3 expression was significantly associated with longer overall survival and time to recurrence especially in advanced TNM stages and Edmondson–Steiner grades.[497] In addition, previous studies demonstrated that Beclin I expression was reduced in human HCC cells especially with apoptotic defect and that HCC patients with positive Beclin 1 expression had a higher tumor differentiation and a significantly better prognosis both in terms of disease-free and of overall survival.[498,499] Moreover, positive cytoplasmic immunostaining of p62, a selective autophagy substrate, was increased in HCC samples compared to the surrounding non-tumorous liver tissue and cirrhotic nodules in HCC patients with or without HCV suggesting that human HCCs are autophagy defective.[500,501]

Furthermore, several *in vitro* studies supported that the autophagic pathway was inhibited in HCC.[458-462] Decreased expression of autophagic genes (Atg5, Beclin 1, and Atg7) and their corresponding autophagic activity has been also demonstrated in HCC cell lines compared with a normal hepatic cell line especially in highly malignant HCC cell lines.[501] Also, expression of Atg5 sensitized tumor cells to chemotherapy, but silencing of Atg5 resulted in a resistance to the chemotherapy.[280] Takamura et al (2011) [502] found that mice with systemic mosaic deletion of Atg5 and liver-specific $Atg7^{-/-}$ develop liver tumors. In these mice, tumor cells originated in autophagy-deficient hepatocytes showed p62 accumulation, mitochondrial swelling and oxidative stress and genomic damage responses. Also, mice with heterozygous disruption of Beclin-1 have a high frequency of spontaneous HCC,[326] while induction of autophagy resulted in apoptotic cell death and inhibited the *in vivo* tumor growth in HCC cell lines and in a murine *in situ* hepatoma model.[500,503]

Despite the literature available, the role of autophagy in hepatocarcinogenesis remains controversial due to its complicated roles in the regulation of cell survival and cell death.[288,336] Many tumor suppressor genes, such as p53, LKT, AMPK, and PTEN, suppress autophagy and many oncogenes, including PI3K, Akt and anti-apoptotic Bcl-2 family proteins are positive regulators of autophagy.[504] Interestingly, dual effects of autophagy in cancer have been proposed. Autophagy might have cancer repressing and, in some situations, cancer promoting effects depending on the tumor microenvironment.[318,333] The findings of the present study suggest that autophagy may function as a tumor suppression mechanism. Autophagy removes damaged proteins and organelles, especially damaged and senescent mitochondria and thus, restricts oxidative and metabolic stress and limits genetic instability and the accumulation of oncogenic

mutations.[318,321] Moreover, Atg5, an autophagy protein cleaved by apoptotic stimuli and subsequently translocated to mitochondria, can trigger caspase activation and apoptotic cell death.[280] In addition, autophagic degradation prevents the accumulation of p62, which was shown to be important in the promotion of tumorigenesis through persistent activation of nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2), deregulation of NFκB, Wnt and mTOR signaling, accumulation of ROS, and increased DNA damage.[330] Also, autophagy promotes expression of cell cycle inhibitors ($p16^{Ink4a}$, $p21^{Cip1}$, and $p27^{Kip1}$) inducing cell senescence and autophagy-mediated tumor dormancy.[329] Autophagy may also protect against tumorigenesis by limiting intratumoral necrosis, chronic inflammation and an increased release of cytokines,[505] increasing tumor antigen presentation for immunosurveillance against tumor cells [313,331] and inhibiting angiogenesis through degradation of neuropilin 1, a positive regulator of VEGF signaling.[332] Defective autophagy, thus, results in a hostile microenvironment favoring malignant transformation and tumor progression.[318]

On the other hand, mounting evidence suggests that autophagy may have cancer promoting effect.[333] Several studies demonstrated that expression of autophagy key regulators (LC3, Beclin-1, ULK1) was increased in HCC compared with non-tumoral parenchyma and was significantly correlated with tumor size, histologic grade, advanced tumor stages, vascular invasion, lymph node metastasis and worse relapse-free and overall survival suggesting that activation of autophagy favors the development and progression of HCC.[506-510] The cytoprotective function of autophagy might enhance the prolonged survival of tumor cells.[333] Autophagy confers stress tolerance, reduces oxidative stress, inhibits apoptosis and provides key intermediates to sustain cell metabolism and thus, serves to enhance cancer cell survival in a less optimal environment.[334.511] The autophagy process has been shown to be activated in the tumor interior rather than in cancer margins, protecting interior tumor cells against stress and cell death under an hypoxic-ischemic environment.[512] Moreover, it has been reported that autophagy is essential for maintenance and survival of liver cancer stem cells.[333] Meanwhile, cancer cells can also use autophagy as a cell survival mechanism against cellular stress or apoptosis induced by many chemotherapeutic drugs.[513] This is particularly crucial because many current cancer therapy agents activate autophagy. [335]

Relationship between mTOR and autophagy in HCV-related liver disease:

The mTOR pathway plays a pivotal role in the regulation of autophagy. mTOR has been identified as down-regulator of autophagy in response to diverse inputs. Inhibition of mTOR is a critical step in autophagy induction.[287] However, the cross talk between mTOR pathway and autophagy during HCV infection remains unknown.[363,369] The present study showed that the intrahepatic expression of the autophagy marker, Atg5, was inversely correlated with serum levels and intrahepatic expression of mTOR in patients with chronic HCV infection. This inverse correlation was also significant when patients with HCV-related HCC were considered. Huang et al (2013) [369] found that inhibition of mTOR activity in Huh7 cells either harboring HCV-N (genotype 1b) full-genomic replicon or infected with JFH1 (genotype 2a) virus, led to the activation of ULK1 and thus to

autophagy induction. Also, knockdown of mTOR significantly enhanced the LC3B-II level and punctate LC3B signals in HepG2 cells suggesting that autophagy is induced by repressing the mTOR expression.[500] Moreover, rapamycin, in addition to inhibiting the mTOR pathway, induced autophagy with an increase in LC3 positive autophagosomes in cultured hepatocytes.[514] Also, rapamycin treatment facilitated autophagy and prevented p62 accumulation with a concomitant NF-κB activation and tumor cell proliferation in a mouse lung model.[515] Nevertheless, autophagy suppression could be one of the mechanisms by which mTOR promotes HCV-related liver injury and HCC development. mTOR may utilize autophagy inhibition as a mean to augment inflammation, fibrosis and cancer cell progression and survival.[287] However, the lack of a correlation between autophagy and hepatic steatosis in the present study may suggest that mTOR-induced lipogenesis was not mediated by autophagy suppression.

The mechanism by which mTOR is involved in the negative control of autophagy is still largely unknown. It has been demonstrated that mTOR activation inhibits autophagy induction via direct phosphorylation of Atg13 at several serine residues causing a reduced binding affinity between of Atg13 and ULK1 and thus inhibition of the membrane targeting of the ULK1 kinase complex and repression of autophagy.[291] Besides regulating the Atg1/ULK complex, mTOR suppresses autophagy via phosphorylation of Tap42, which activates the catalytic subunits of the serine/threonine protein phosphatase 2A, a negative regulator of autophagy.[516] Also, mTOR inhibits p73, a member of p53 family, which regulates the expression of several Atg genes including Atg5 in response to different cellular stresses.[292] Moreover, a new substrate for mTOR known as the death-associated protein 1, which is phosphorylated by mTOR, leads to autophagy inhibition.[517] In addition, mTORC2, through activation of AKT, inhibits of the transcription factor FoxO3, which is able to suppress the expression of autophagy genes.[518]

However, the present study demonstrated that a group of patients with HCV-related liver disease showed simultaneous high expression (score 2 or 3) of both mTOR and Atg5 in hepatocytes. This unexpected finding indicates that autophagy was induced despite mTOR activation in these patients. Similarly, Shrivastava et al (2012) [369] found that Beclin1, an autophagy marker, was upregulated in HCV-infected hepatocytes with increased expression of total mTOR, p-mTOR and its substrate, phospho-4E-BP1. Moreover, a recent study showed that inhibition of the mTOR pathway could not completely reverse autophagy suppression in HCV-infected cells suggesting that other mechanisms independent of mTOR could be at work to regulate autophagy.[363] These discordant results may suggest that the status of autophagy activity in HCV infection might be determined by the balance between mTOR activation as an autophagy suppressor and the effect of autophagy inducers (HCV proteins, ER stress, ROS and inflammation) in HCV-infected cells.[519] It has been demonstrated that autophagy induction may be mediated by the class III PI3K, Vps34, which is also known to be involved in activation of mTOR.[520] Vps34, together with its regulatory component p150 (Vps15 in yeast), forms 2 different complexes, with complex I (containing Atg6/Beclin 1 and Atg14) functioning in the autophagy pathway.[265] Moreover, HCV-mediated autophagy may act as an upstream positive regulator of mTOR signaling pathway. A recent study showed that a decreased expression of phospho-mTOR, total mTOR and 4E-BP1 was observed in autophagy-

knockdown HCV-infected cells.[369] In addition, it has been shown that the S6K, a downstream target of mTOR, may enhance the stimulation of autophagy, although it is not mandatory for its initiation. The S6K may contribute to the basal activity of autophagy via its feedback inhibition of the class I, PI3K-dependent insulin signaling pathway which inhibits autophagy.[521] Thus, mTOR signaling and autophagy are inversely inter-related or act concurrently in HCV infection. A potential explanation of this paradox is likely that HCV infection is inducing autophagy for establishment of infection, while activating mTOR signaling for hepatocyte growth.[363]

SUMMARY

Hepatitis C virus (HCV) infection is a well-established cause of wide range of liver diseases starting from acute hepatitis passing into chronic hepatitis, liver cirrhosis and finally hepatocellular carcinoma (HCC). Disease progression occurs as a result of viral persistence which leads to perpetuation of inflammation, fibrogenesis and hepatocarcinogenesis. These occurs secondary to the direct effect of viral coded proteins as well as modulation of cell cycle and cellular biological activities through genetic and epigenetic alterations and dysregulation of multiple signal transduction pathways. Mammalian target of rapamycin (mTOR), a 289-kDa serine/threonine protein kinase, is a member the phosphoinositol 3-kinase (PI3K)-related kinase family. The mTOR signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of various cellular processes including cell metabolism, growth, proliferation, and survival and cell cycle progression. In addition, mTOR is a down-regulator of the intracellular process of autophagy in response to cellular physiological conditions and environmental stress. Autophagy is a catabolic process important in organelle degradation and protein turnover required to provide biological material so as to sustain anabolic processes. Beyond maintaining cellular homeostasis, autophagy plays an important role in cell quality control, energetic balance, remodeling, and defense against extracellular insults and pathogens. It is likely that dysregulation of the mTOR-autophagy pathway may contribute to many human disorders including viral infections, inflammatory disorders and cancer and could be an attractive avenue for future therapeutic approaches.

Therefore, the present study was designed to study the role of mTOR and autophagy in the progression of HCV-related liver disease.

To achieve this goal, the present study included 54 treatment-naïve patients with HCV-related liver disease referred to the Main Alexandria University Hospital. They were 27 patients with chronic hepatitis C (CHC) (12 males and 15 females); 13 cirrhotic patients without HCC (10 males and 3 females) and 14 cirrhotic patients with HCC who underwent surgical resection of the tumor (9 males and 5 females). The diagnosis of chronic HCV infection was based on seropositivity of circulating HCV antibodies, detectable serum HCV RNA and histopathological characteristics. The presence of cirrhosis was determined by clinical, biochemical and ultrasonographic evidences and/or histopathological examination. The diagnosis of HCC was based on serum levels of alpha fetoprotein (AFP), ultrasonography/ triphasic computed tomography (CT) and when needed, dynamic magnetic resonance imaging and was confirmed by histopathological examination of surgically-resected tumors. Patients with HCV-related liver disease were excluded from the study if they had seropositivity for hepatitis B virus infection; history of alcohol consumption; other known causes of chronic liver disease; concomitant schistosomiasis; hepatic decompensation, bleeding diathesis; chronic diseases such as diabetes mellitus, connective tissue diseases or other autoimmune diseases; other infections; other malignancy; cardiac, respiratory or renal disease and previous antiviral treatment or locoregional or systemic therapy for HCC. Also, 15 age- and sex-matched healthy subjects with no evidence of liver disease were included in the study.

All patients included in the study were evaluated clinically as regards the apparent duration and possible risk factors of HCV infection, symptoms and signs of chronic liver disease (right hypochondrial pain, jaundice, ascites, hepatic encephalopathy, previous gastrointestinal bleeding), liver and spleen sizes and the presence of palpable focal hepatic lesions. Ultrasonographic and triphasic CT examination was performed for assessment of liver echopattern (normal, bright or coarse), liver and spleen size, presence of ascites and tumor characteristics in patients with HCC (maximum diameter; number of nodules; location and extension as % of the liver). Blood samples were collected from all patients on admission and from healthy subjects and the following tests were performed: complete blood picture, liver test profile [serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), serum albumin, serum bilirubin, serum gamma glutamyl transpeptidase (GGT), and prothrombin activity (PA)/international normalized ratio], serum creatinine and serum AFP levels using standardized enzyme linked immunosorbant assay (ELISA) kit. The severity of liver disease in patients with HCV-related cirrhosis with and without HCC was graded according to Child-Pugh classification and the Model for End Stage Liver Disease (MELD) score. The staging of HCC was determined according to the Barcelona Clinic Liver Cancer (BCLC) and the Cancer of the Liver Italian Program (CLIP). The mTOR protein levels in sera of patients with HCV-related liver disease and healthy subjects were measured using commercially-available human solid phase sandwich ELISA kit.

Liver biopsy was performed in 30 patients with chronic HCV infection (all patients with CHC and three cirrhotic patients without HCC). Representative samples of surgically-resected HCCs and surrounding non-neoplastic liver tissues were also obtained. Histopathological examination was done to assess the histological activity grade (A0-A3) and fibrosis stage (F0-F4) according to the METAVIR scoring system and the grade of steatosis (0 = absent; 1 = mild, less than one third; 2 = moderate, one third to two thirds and 3 = marked, more than two thirds) for liver specimens from patients with with chronic HCV infection and the surrounding non-neoplastic liver tissues. The diagnosis of HCC was confirmed by histopathological examination and the tumor was graded according to Edmonson and Steiner's criteria. Immunohistochemical staining of liver specimens was done using anti-human antibodies against mTOR and Atg5 (as a marker of autophagy). Immunostaining results for mTOR and Atg5 were evaluated semi-quantitatively according to the percentage of positively-stained cells in non-overlapping microscopic fields. Both cytoplasmic and nuclear staining was assessed for mTOR expression, while cytoplasmic staining was assessed for Atg5 expression. The percentage of positively stained cells was determined by counting a minimum of 100 cells, and the average counts were recorded, and scored as follows: 0 = absent staining; 1 = weak, < 10% of cells were positive; 2 = moderate, 10-50% of cells were positive or 3 = strong, > 50% of cells were positive.

Statistical analysis of the data was performed using SPSS program (version 20.0) (IBM-SPSS Inc., New York, United States) for Windows. Statistical significance was assessed at $P < 0.05$. The results from the present study are summarized as follows:

- Mild to moderate hepatomegaly with bright echopattern was found in 16 (59.3%) patients with CHC, while in cirrhotic patients without HCC, the liver was enlarged in 5

(38.5 %) patients, normal in 3 (23.1 %) patients and shrunken in 5 (38.5 %) patients with coarse echopattern in 10 (76.9 %) patients.

- In patients with HCC, the liver was enlarged in 10 (71.4%) patients and focal hepatic lesions were clinically palpable in 7 (50 %) patients. The maximum diameter of HCC ranged between 2.7 and 11.6 cm with a mean value of 6.19 ± 2.93 cm. The tumors were uninodular in all patients and were located in the right lobe in 5 (35.7 %) patients and in the left lobe in 9 (64.3%) patients. The extension of all surgically-resected HCCs was < 50% of the liver. Serum AFP levels ranged between 11-20973 ng/ml in patients with HCC.

- The serum HCV-RNA level ranged between 19-6500 $x10^3$ IU/ml (mean ± SD = 923.27 ± 1504.44 $x10^3$ IU/ml) in patients with CHC, between 52.6 - 6231 $x10^3$ IU/ml (mean ± SD = 1543.53 ± 2208.21 $x10^3$IU/ml) in cirrhotic patients without HCC and between 110-2300 $x10^3$ IU/ml (mean ± SD = 675.00 ± 626.42 $x10^3$ IU/ml) in patients with HCC.

- According to Child-Pugh classification**,** cirrhotic patients without HCC were classified as class A in 10 (76.9%) patients and class B in 3 (23.1%) patients and patients with HCC were classified as class A in 12 (85.7%) patients and class B in 2 (14.3%) patients. The MELD score ranged between 6-9 (mean ± SD = 7.62 ± 0.87) in cirrhotic patients without HCC and between 6-10 (mean ± SD = 8.14 ± 1.46) in patients with HCC.

- The staging of HCC was classified as stage A2 in 7 (50.0%) patients, stage A3 in 2 (14.3%) patients and stage B in 5 (35.7%) patients according to the BCLC staging system. The CLIP scoring system categorized patients with HCC as CLIP stage 0 in 5 (35.7%) patients, CLIP stage 1 in 7 (50.0%) patients and CLIP stage 2 in 8 (14.3%) patients.

- The serum mTOR levels ranged between 0.8-4.4 ng/ml in patients with CHC, between 1.5-4.5 ng/ml in cirrhotic patients without HCC, between 3.7-9.5 ng/ml in patients with HCC and between 0.6-1.6 ng/ml in healthy subjects. The mean serum mTOR levels was significantly higher in patients with CHC, cirrhotic patients without HCC and patients with HCC than in healthy subjects (2.22 ± 1.20 ng/ml, 3.49 ± 0.79 ng/ml and 6.19 ± 1.63 ng/ml vs 1.13 ± 0.30 ng/ml respectively), in cirrhotic patients with and without HCC than in patients with CHC and in cirrhotic patients with HCC than in those without HCC ($P < 0.001$).

- By plotting receiver-operating characteristic (ROC) curve, the sensitivity and specificity of serum mTOR levels in discriminating cirrhotic patients with and without HCC were 92.9% and 100% respectively at a cut-off value of 4.55 ng/ml [Area under the curve (AUC) = 0.970].

- According to METAVIR scoring system, patients with chronic HCV infection showed histological activity grade A1 in 10 (33.3%) patients, A2 in 13 (43.3%) patients and A3 in 7 (23.3%) patients while fibrosis stage was classified as F1 in 7 (23.3%) patients, F2 in 15 (50.0%) patients, F3 in 5 (16.7%) patients and F4 in 3 (10%) patients. Steatosis

was absent in 5 (16.7%) patients, mild in 10 (33.3%) patients, moderate in 8 (26.7%) patients and marked in 7 (23.3%) patients.

- According to Edmondson and Steiner's grading system, HCV-related HCCs were graded as grade II in 7 (50.0%) patients, grade III in 2 (14.3%) patients and grade IV in 5 (35.7%) patients.

- The surrounding non-neoplastic liver tissues showed cirrhosis (METAVIR F4) in all patients with HCC. The histological activity grade was A2 in 4 (28.6%) patients and A3 in 10 (71.4%) patients. Steatosis was mild in 2 (14.3%) patients, moderate in 11 (78.6%) patients and marked in one (7.1%) patient.

- Positive immunostaining for mTOR was detectable as cytoplasmic and/or nuclear staining in 18 (60.0%) of patients with chronic HCV infection; of them, 4 (13.3%) patients showed weak staining, 6 (20.0%) patients showed moderate staining and 8 (26.7%) patients showed strong staining.

- In HCV-related HCCs, positive mTOR immunostaining was detected in 12 (85.7%) patients; of them weak staining was found in one (7.1%) patient, moderate staining in one (7.1%) patient and strong staining in 10 (71.4%) patients. The surrounding non-neoplastic liver tissues also showed positive mTOR immunostaining in 12 (85.7%) patients; among them, weak staining was found in 3 (21.4%) patients, moderate staining in 6 (42.9%) patients and strong staining in 3 (21.4%) patients.

- There was a significant difference in mTOR staining intensity among patients with HCV-related liver disease ($P = 0.032$). The mTOR staining intensity was significantly higher in HCV-related HCCs than in the chronic HCV infection tissues ($P = 0.049$) and the surrounding non-neoplastic liver tissues ($P = 0.035$) while no statistically significant difference was found between the chronic HCV infection tissues and the surrounding non-neoplastic liver tissues ($P = 0.235$)

- Positive cytoplasmic immunostaining of Atg5 was detectable in 28 (93.3%) patients with chronic HCV infection; one (3.3%) patient showed weak staining, 10 (33.3%) patients showed moderate staining and 17 (56.7%) patients showed strong staining.

- In HCV-related HCCs, positive Atg5 immunostaining was detected in all patients; weak staining in 5 (35.7%) patients, moderate staining in 5 (35.7%) patients and strong staining in 4 (28.6%) patients while the surrounding non-neoplastic liver tissues also showed positive Atg5 immunostaining in all patients; among them, moderate staining in 6 (42.9%) patients and strong staining in 8 (57.1%) patients.

- There was a significant difference in the Atg5 staining intensity among patients with HCV-related liver disease ($P = 0.038$). The Atg5 staining intensity was significantly lower in HCV-related HCCs than in the chronic HCV infection tissues ($P = 0.019$) and the surrounding non-neoplastic liver tissues ($P = 0.046$) while no statistically significant

difference was found between the chronic HCV infection tissues and the surrounding non-neoplastic liver tissues ($P = 0.840$).

- Simultaneous high expression (score 2 or 3) of both mTOR and Atg5 was detectable in 17 (38.6%) patients; in hepatocytes of 11 (36.7%) patients with chronic HCV infection and in tumor cells in 6 (42.9%) patients with HCC.

- Statistical correlations between serum mTOR levels, intrahepatic expression of mTOR and Atg5 and other parameters in patients with chronic HCV infection showed the following results:

  - No statistically significant correlations were found between serum mTOR levels, and the expression of mTOR and Atg5 on one hand and age and serum levels of GGT and serum HCV RNA levels on the other hand ($P > 0.05$).

  - The serum mTOR levels and intrahepatic mTOR expression showed significant positive correlations with serum AST levels ($P < 0.001$ and $P = 0.001$ respectively), serum ALT levels ($P < 0.001$ and $P = 0.005$ respectively), the METAVIR histological activity grade ($P = 0.039$ and $P = 0.015$ respectively) and fibrosis stage ($P = 0.002$ and $P = 0.006$ respectively) and steatosis grade ($P < 0.001$ and $P = 0.032$ respectively).

  - The intrahepatic Atg5 expression showed significant inverse correlations with serum AST and ALT levels ($P = 0.025$ and $P = 0.021$ respectively) and the METAVIR histological activity grade and fibrosis stage ($P = 0.001$ and $P = 0.021$ respectively). There was no significant correlation between intrahepatic Atg5 expression and steatosis grade ($P > 0.05$).

  - The serum mTOR levels and intrahepatic mTOR expression were positively correlated ($P = 0.004$) and both showed inverse correlations with the intrahepatic Atg5 expression ($P = 0.029$ and $P < 0.001$ respectively).

- Statistical correlations between serum mTOR levels, intrahepatic expression of mTOR and Atg5 and other parameters in patients with HCV-related HCC showed the following results:

  - No statistically significant correlations were found between serum mTOR levels, and the expression of mTOR and Atg5 in HCCs on one hand and Child-Pugh and MELD scores and the expression of mTOR and Atg5 in the surrounding non-neoplastic liver tissues on the other hand ($P > 0.05$).

  - The serum mTOR levels and mTOR expression in HCCs showed significant positive correlations with serum AFP levels ($P = 0.003$ and $P = 0.003$ respectively), HCC maximum diameter ($P = 0.018$ and $P = 0.032$ respectively), CLIP stage ($P = 0.032$ and $P = 0.002$ respectively) and HCC histological grade ($P = 0.003$ and $P = 0.025$ respectively).

- The Atg5 expression in HCCs showed significant inverse correlations with serum AFP levels (P = 0.004), HCC maximum diameter (P < 0.001), BCLC stage (P = 0.002), CLIP stage (P< 0.001) and HCC histological grade (P < 0.001).

- The serum mTOR levels and mTOR expression in HCCs were positively correlated (P = 0.033) and both showed inverse correlations with the Atg5 expression in HCCs (P = 0.005 and P < 0.001 respectively).