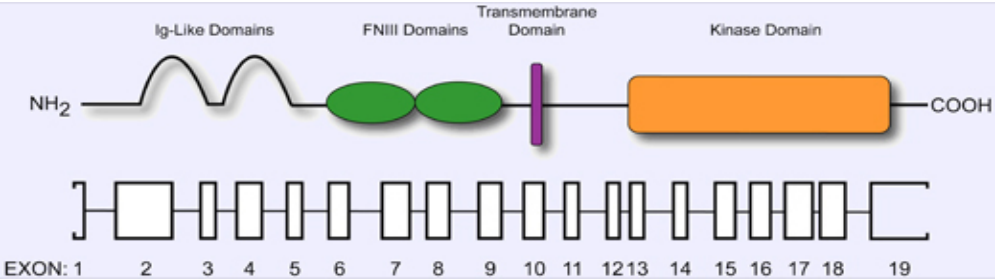
UNIVERSITÀ DEGLI STUDI DI PALERMO

MEDICINA MOLECOLARE – INDIRIZZO BIOPATOLOGIA
Dipartimento di Biopatologia e Biotecnologie Mediche
Settore Scientifico Disciplinare BIO-13

Correlation between the single nucleotide polymorphism (SNP) rs4374383 in MERTK gene with the risk of development and progression of liver disease



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“Non siamo noi a scegliere quali tempeste debbano far parte della nostra vita ma di affrontarle con grinta e tenacia SI! ”Un grazie speciale alla mia Famiglia e soprattutto alla mia Mamma che oggi più che mai è il mio pilastro di vita.

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CHAPTER 1
General Introduction

1.1 APOPTOSIS AND FIBROGENESIS

Apoptosis is a form of cell death and is characterized by membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr JF, et al., 1972). The fragmentation of the cell into the membrane is defined by apoptotic bodies. It is an essential process that plays a critical role in tissue homeostasis, embryologic development, immunity and resolution of inflammation.

The clearance of apoptotic bodies is named efferocytosis and is mediated by professional phagocytes, such as macrophages and immature dendritic cells (DC), that are mobile and capable of infiltrating a wide variety of tissue (Ablin J, et al., 2005), performing key roles during the inflammation and its resolution.

The recognition of apoptotic cells and their efferocytosis by professional phagocytes leads to secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, which inhibit the production of inflammatory mediators (Voll RE, et al., 1997; Fadok VA, et al., 1998). The defective clearance of the apoptotic cells is associated with many autoimmune and chronic inflammatory disorders, being the result of genetic anomalies or the persistent diseases state, and contributes to their the establishment and progression (Jang E, et al., 2013).

The initial event in phagocytosis is the recognition of the target; the successful engulfment requires that apoptotic cells must generate two signals: “a find me” signal and an “eat me” signal.

Generally, the apoptotic cell sensing system recognizes the change of expression of lipids or carbohydrates on the outside membrane of dying cells (Greenn D, 2003; Ravichandran KS, and Lorenz U, 2007). Indeed, the loss of membrane asymmetry during the apoptosis leads to oxidation of phospholipids, that represent an “eat-me” signal, recognized by specific brinding molecules (or find me signals) that facilitate the phagocytosis through interaction with the specific receptors (Fadok VA. et al.; 1998).

Once recognized as “eat me” signal, the phosphatidylserine is traslocated from cell surface (Savill J, et al., 2000). The phagocytes recognize this signal and trasduce him to the cell machinery required for engulfment. (Henson PM et al., 2001). Finally the ingested particles enter in the lysosomal system and are degradated.

The ingestion of the apoptotic cells results in potent anti inflammmatory and immunosuppressive effects, through the production of anti-inflammatory cytokines and the suppression of pro-inflammatory mediators release from activated macrophages (Voll RE, et al., 1997, Fadok VA, et al., 1998; Lemke G, and Rothlin C, 2008).

The professional phagocytes display several receptors on the membrane that participating in the recognition and in the efferocytosis processes. The involved receptors include: scavenger receptors class A, CD36 and phosphatidylserine (PS) receptors, molecules belonging to the immunoglobulin superfamily, vitronectin receptor, CD14;lipopolysaccharide (LPS) receptor, complement receptor and TAM receptors (Platt N, et al., 1996; Taylor PR, et al., 2000).

TAM receptors are one of 20 subfamilies of receptor tyrosine kinases (RKTs) that regulate different processes including cellular proliferation/survival, cellular adhesion/migration and release of the inflammatory cytokines.

This subfamily of RKTs comprises three receptors, Tyro-3, Axl and MerTK, which share the ligands Gas6, TULP1, Tubby and protein S, and mediate several cellular functions, including macrophage clearance of the apoptotic cells, platelet aggregation and cellular differentiation of natural killer (NK) In particular, MerTK receptors seems to be essential for engulfment and efferocytosis (Scott Rona S, et al., 2001).

The efficient execution of efferocytosis is a key mechanism in maintaining tissue homeostasis and also contributes to the initiation of repair processes following an injury (Rigamonti E, et al., 2014; Wan E, et al., 2013).

The recognition of apoptotic cells themselves, by professional phagocytes, can reinforces pathways and enhances efferocytic ability through the activation of Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), PPAR-δ and Liver X Receptor (LXR) followed by the enhanced CD36 and MerTK expression (Gregory CD, et al.; 2004; McColl A, et al.; 2009) and the secretion of efferocytic bridge molecules (A-Gonzalez N, et al., 2009; Mukundan L, et al., 2009; Roszer T, et al., 2011).

PPAR-γ, PPAR-δ and LXR are also up-regulated both during IL-4 signaling (Fernandez-Boyanapalli RF, et al., 2009), that stimulates TGF-β production, and during the macrophage differentiation (Mukundan L, et al.; 2009; Roszer T, et al.; 2011); these events culminate in the suppression of the inflammation.

These observations reinforce the concept of plasticity in macrophage programming, during the course of the inflammatory response, with the early induction of protective properties and later development of activities that promote the resolution.

The apoptotic response is associated with liver disease and includes soluble stimuli, inflammatory cells, resident parenchymal cell and fibrogenic cells. The cellular apoptosis is the first response to toxic events, following a viral hepatitis, alcohol-induced disease and nonalcoholic steatohepatitis, and correlates with disease severity and hepatic fibrosis (Natori S, et al., 2001; Feldstein AE, et al., 2003).

In the liver, the hepatic stellate cells (HSCs), the pericytes of the sinusoids and the kupffer cells are able to perform efferocytosis (Canbay A, et al., 2003; Jiang JX, et al., 2009) and contribute to inflammation through the activation of several mechanisms. First, if apoptosis is activated improperly, it leads to accumulation of the apoptotic cells and to generation of soluble mediators that elicit the inflammatory response (Patel T, et al., 1998). Alternatively, the apoptosis of the hepatocyte induces the production of the chemokines, througha mechanism Fas/Fas-ligand-

dependent, that promotethe infiltration of the neutrophilsand stimulate the inflammation (Faouzi A, et al., 2001).

A consequence of the hepatic inflammation is the activation of HSCs into a myofibroblast–like phenotype (Maher JJ, et al., 2001) which directly stimulate fibrogenesis (Fadok VA, et al., 1998; Platt N, et al., 1998).This transitionis responsible of extracellular matrix production within the injured liver(Friedman SL, 1993).

HSCs express as core machinery to phagocytose the apoptotic bodies the PS receptor, that is an “eat me signal” to stimulate the engulfment (Somersan S, et al., 2001). When the HSCs engulfe apoptotic bodies, a switch from quiescent cells to myofibroblasts is observed, leading to the production of TGF-β (profibrogenic cytokine), and collagen alpha I (the major constituent of cirrotic liver) (Canbay A, et al., 2003). To provoke engulfment, apoptotic cells release into the extracellular space chemical mediators that serves as chemo-attractant signal to recruit professional phagocytes to the site of the dying cell. These chemical mediators may function also to recruit HSCs to sites of liver injury and promote their activation (Bourd-Boittin K, et al., 2009).

Several studies have linked hepatocyte apoptosis and efferocytosis to liver fibrosis (Feldestein AE, et al., 2003; Meriden Z, et al., 2010). The hepatocyte apoptosis is generally mediated by the extrinsic pathway that involves four death receptors, such as the FAS, the tumor necrosis factor receptor 1 (TNF-R1), the death receptor 4 and 5 (DR4 and DR5) and their ligands, Fas ligand (FasL) the TNF-α and the TRAIL (Faubion WA, et al., 1999).

The Fas and TNF-R1activation is associated with hepatocyte apoptosis in a wide variety of liver disease, including viral hepatitis, nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Akazawa Y, et al., 2007). After the engulfment, the professional phagocytesproducts the death ligands (TNF-α, TRAIL and FasL). In addiction the apoptotic DNA is recognized by intracellular Toll-Like Receptor 9 (TLR-9), which activates a variety of signaling, leading to the production ofcytokines, the amplification of the inflammatory responseand the expression of TAM receptors on the surface of phagocytes (Luke AJ, et al., 2007).

HSCs, juxtaposed to the hepatocytes, directly stimulate the fibrogenesis (Caberoy NB, et al., 2010; Canbay A, et al., 2003; Lauber K, et al., 2003) being located to the engulfment site(Canbay A, et al., 2004). In contrast, kupffer cells, the resident macrophages, are located within the sinusoids and must to migrate into the liver parenchyma for have access to the apoptotic bodies (Canbay A, et al., 2003).

The HSCs produceand increase the expression of potent pro-fibrogenic cytokines, such as TGF-β, and of type I collagen through the MAP/PI-3K pathway (Canbay A, et al., 2003) and are functionally involved in the progression of liver fibrosis (Patin E, et al., 2012).

1.2 TAM RECEPTORs

The TAM receptors are one of 20 subfamilies of RTKs (Robinson DR, et al., 2000) and include three members, Tyro-3, Axl and MerTK. These receptors are characterized by an extracellular region containing two immunoglobulin (Ig) related domains, followed by two fibronectin type III (FN-III) related repeats, a single transmembrane domain and a cytoplasmic tyrosine kinase moiety, followed by a short C-terminal tail containing a few tyrosine residues (*Fig.1*).

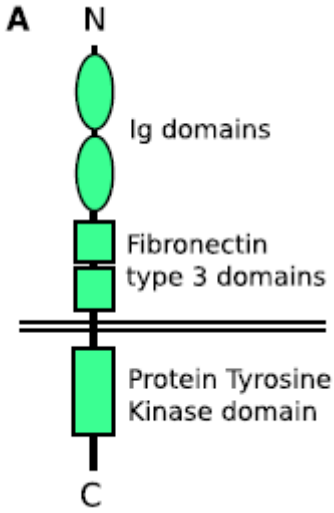

Fig. 1. The structure of TAM receptors.

Generally, these receptors exist as monomers on the cytoplasmic membrane and the binding of their ligands leads to dimerization and subsequent trans-autophosphorylation of tyrosine residues (Hubbard SR, et al., 1998). Once the receptor is activated, the tyrosines phosphorylated provide the docking sites for the downstream signaling components (Sasaki T, et al., 2006).

In many cells, the activation of these RTKs is coupled to the downstream activation such as the phosphoinositide 3 kinase (PI3K)/AKT pathway, the ERK1/2 Ras and the MAP kinase signaling (Keating AK, et al 2010; Lijnen HR, et al., 2011; Ou WB, et al., 2011) (*Fig. 2 A*).

Macrophages, dendritic cells and other sentinel cells of immune system express also cytokine receptor signaling systems, e.g. the type I interferon receptor (IFNR), that are directly coupled to TAM receptors and codependent on them. In these cells, the TAM-activated PI3K/AKT pathway is often dominated by a stronger TAM-activated JAK/STAT inhibitor signaling (Zong C, et al., 1996; Rothlin CV, et al., 2007; Lemke G, and Rothlin CV, 2008) (*Fig. 2 B*).

The differential TAM activation of PI3K/AKT versus JAK/STAT signaling may be important for the different activation of distinctactivities TAM-regulated (*Fig.2*).

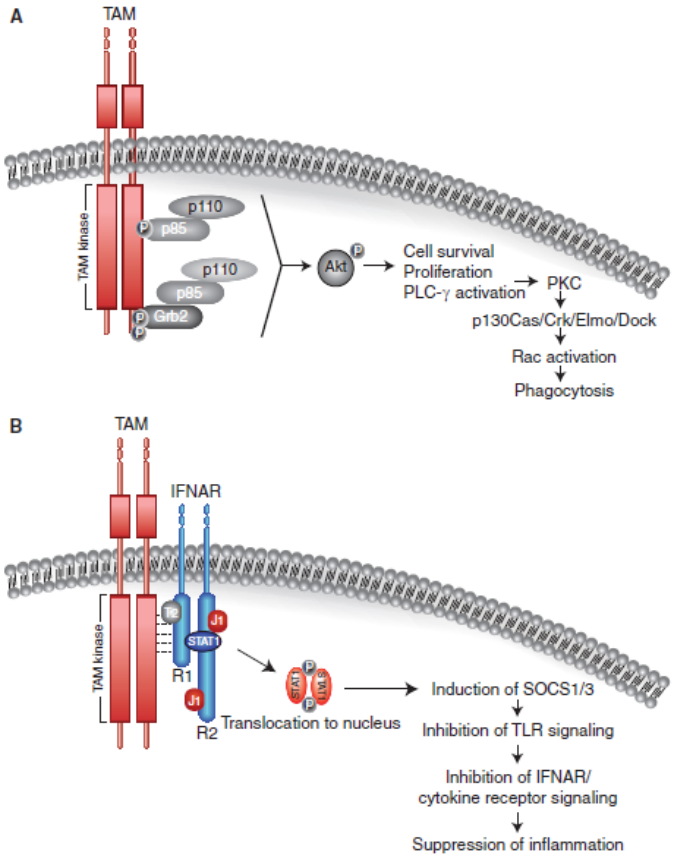

Figure 2 -A and -B. TAM receptor signaling pathways.

Growth-arrest specific 6 (Gas6), Protein S, Tubby and Tubby-like protein 1 have been identified as ligands for TAM receptors.

Gas6 is a 75-kDa vitamin K-dependent protein, expressed from endothelial cells (Manfioletti G, et al., 1993), vascular smooth muscle cells (Nakano T, et al., 1995) and bone marrow (Avanzi GC, et al., 1997). It is barely produced in the liver, but instead in the heart, kidneys and lungs.

Protein S is a vitamin K-dependent protein, produced by a variety of cells types (e.g. hepatocytes, endothelial cells, megakariocytes and osteoblasts) (Maillard C, et al., 1992). It is capable of binding Factor Xa (FXa) and FVa, inhibiting directly the coagulation (Heeb MJ, et al., 1993; Heeb MJ, et al., 1994).

Gas6 and protein S show high structural homology with an amino-terminal-γ-carboxyglutamic acid (GLA) domain, four epidermal growth factor-like domains and a carboxy-terminal (C-terminal) region that consists of 2 laminin G repeats (LG1 and LG2) (*Fig. 3*).

These ligands bindthe phosphatidylserine of apoptotic cells, with their N-terminal GLA domain, and the MerTK of macrophages (or Axl and Tyro 3 on dendritic cells), with their C-terminal region, causing the intracellular phosphorilation of the kinase domain (Scott RS, et al., 2001; Todt JC, et al.; 2004).

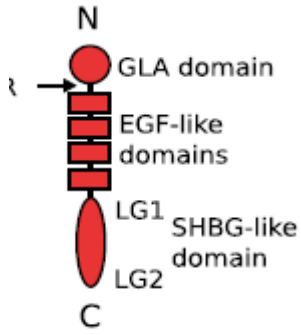

Figure 3. The structure of Gas 6 and Protein S ligands.

The TAM receptors through Gas6 amplifiethe activation of pro-inflammatory endothelial cells (EC) that leads to the expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). Thereby, TAM receptors support the extravasation of the leucocytes, the inflammation and an adhesion of platelets to endothelial cells (Tiwa M, et al., 2008).

Protein S levels are increased in atherosclerotic vessels. Through the activation of MerTK, the protein S inhibits the uptake of low density lipoprotein, by the macrophage scavenger receptor, reducing the formation of foam cells (Liao D,et al., 2009). These mechanisms can explain part of the recent findings about the SNP mutations in the TAM genes, that are related to the formation of atherosclerotic plaques (Hurtado B, et al., 2010; Hurtado B, et al., 2011).

In contrast, the signaling of TAM receptors can also inhibit the inflammation through multiple mechanisms. One of this involved the activation of MerTK that induces the transcription of the anti-inflammatory suppressor of cytokine signaling protein 1 (SOCS1) and SOCS3, inhibitingthe cytokine receptors and the TLR signaling (Rothlin CV, et al., 2007; Sun B, et al., 2010). Another important mechanism is the amplification of efferocytosis and the production of IL-10 and TGFβ (Vandivier RW, et al., 2006).

Recently, two new ligands ofTAM receptorshave been described: Tubby which binds specifically MerTK and Tubby-like protein 1 (TULP1) which interacts with all three RKTs (Caberoy NB, et al., 2010).

Tubby and TULP1 belong to tubby protein family and are bridge molecules that present a N-terminal region(MerTK- binding domain, MPD), and a C- terminal region (phagocytosis pre-binding domain, PPBD) (Carroll et al., 2004). They are able to stimulating macrophage phagocytosis through the MerTK activation (Finnemann SC, 2003).

Mutations in the MerTK gene or, in general, in the TAM genes, that bring to the complete or partial impairment of the phagocytosis, promote the accumulation of apoptotic cells and elicit the pro-inflammatory immune responses. These events can lead to severe states of cellular degeneration and death, suggesting a role of these receptors to regulate the efferocytosis and to maintain the tissue and cellular homeostasis (Lu Q, et al., 1999; Lu Q, and Lemke G, 2001).

1.3 MACROPHAGE POLARIZATION

The macrophages are mononuclear phagocytes, derived from monocytes released from bone marrow into the circulation, which differentiate intothe tissues. They are recognized as heterogeneous cells, since they are critical effectors and regulators of the innate and the adaptative immunity, the systemic metabolism, the angiogenesis, the malignancy and the reproduction (Bolego C, et al., 2013; Roszer T, et al., 2013; Hume DA, 2008).

They play an important role in the tissue homeostasis and the lesions: promote the initiation and progression of tissue injury and improve also the tissue remodeling in various diseases from different etiology (Mantovani A, et al.; 2013; Duffield JS, et al.; 2005).

The macrophage heterogeneity probably reflects the plasticity and the versatility of these cells in response to the exposure to microenvironmental signals. Differential production of cytokines is a key feature of polarized macrophages to specific functional programs that are defined as M1 or M2.

In response to microbial products or interferon-γ (IFN-γ) these cells are activated as M1 macrophages (or classically activated macrophages). M1 cells are characterized by high ability to present antigen, to produce pro-inflammatory cytokines (e.g. interleukin-12, IL-12) and Tumor necrosis factor (TNF), leading to consequent activation of type I polarized response (Th1) (*Fig. 4*).

In contrast, various signals, e.g. IL-4, IL-13, glucorticoids and IL-10, immunoglobulin complex/TLR ligands, induce the distinct M2 functions (or alternatively activated macrophages). M2 cells are able to activate the inflammatory response adaptive Th2, to scavenge the debris, to promote the angiogenesis, the tissue remodeling and the repair (Mantovani A, et al.; 2004; Mantovani A, et al., 2002) (*Fig. 4*).

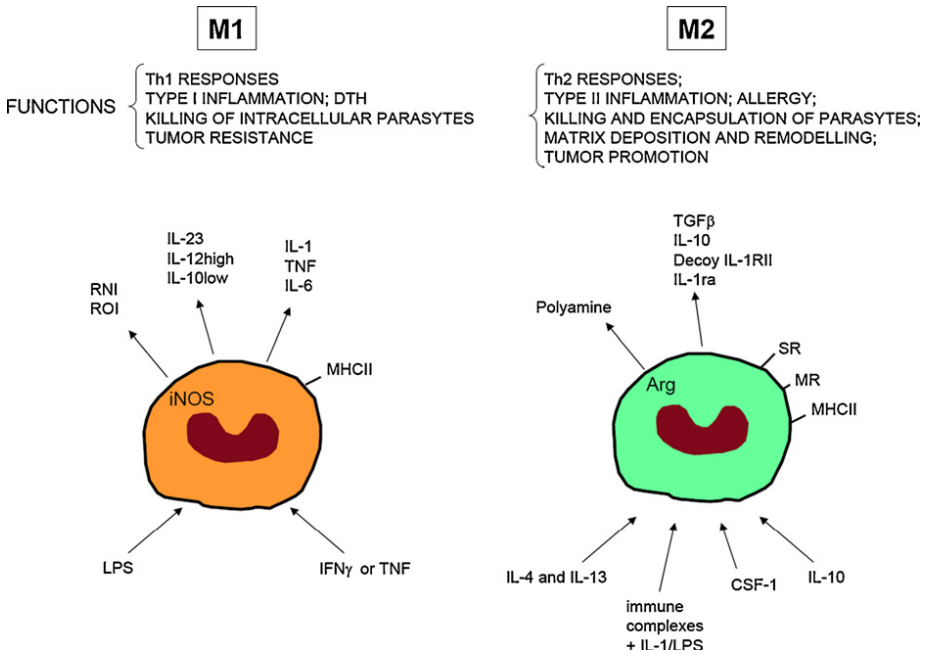

Figure 4. Fuctions of M1 and M2 polarized macrophages.

In agreement with an alternative activation program, the M2 macrophages express also high levels of scavenger receptor-A (SRA), mannose receptor (MR) (Alalvena et al.; unpublished observations) andTAM receptors. These receptors contribute collectively to regulate the phagocytosis and MerTK plays a predominant role in M2 cells and in thymic macrophages. Mice mutant in Mertk gene were not able to clean efficiently the debris or the apoptotic cells. This deficiency is attributed to macrophages as demonstrated *in vitro* (Scott RS, et al.; 2001).

MerTK receptor is not homogenously distributed in the macrophage population, but is mostly restricted to a subset of M2 macrophages, recently named M2c, that secrete anti-inflammatory cytokines as IL-10 and TGF-β (Martinez FO, et al.;2008).

M2c polarization is closely associated with the up-regulation of MerTK and is required to obtain the macrophages highly specialized in the efferocytosis. M2c cells are also able to release Gas6, that can amplify the secretion of IL-10 in an autocrine manner, via MerTK signaling (Zizzo G, et al.; 2013).

Interestingly, following the engulfment of apoptotic cells, the macrophages activate the tolerogenic pathways to prevent immune responses against self antigens (Henson P, and Hume D, 2006; Taylor PR, et al.; 2005). The tolerance is realized through several mechanisms, including the suppression of inflammatory cytokine production and the release of anti-inflammatory cytokines, as IL-10 and TGF-β (Fadok VA, et al.;1998; Li M, and Flavell R, 2008). If the phagocytosis or the tolerogenic pathways are impaired, the chronic accumulation of free apoptotic material can have pathological consequences (Henson P, and Hume D, 2006; O'Shea J, et al.; 2002). In addition, TAM receptors, able to suppress the TLR signaling, represent a mechanism by which the efferocytosis is coupled to immune tolerance (Lemke G, and Rothlin C, 2008; Rothlin CV, et al., 2007).

Recently, it has been investigated the role of the M2 differentiation in regulating the inflammation and the fibrogenesis (Smith W, et. al.; 1998; Verreck FA, et al.; 2004; Gordon S, 2003). The factors that control these processes are still to understand (Gordon S, 2003).

Zizzo and collegues have performed the first systematic study about the effects ofcytokines and growth factors on human macrophagesphenotype, on MerTK expression and on Gas6secretion. They found that the levels of MerTK and Gas6 followed the expression pattern of CD16 and CD163, molecules identifiable as specific M2c markers (Zizzo G, et al.; 2012).

In addition, M2 cells show also pro-tumoral functions, by promoting the tumor cell survival, proliferation and dissemination (Mantovani A, et al., 2002; Pollard JW, 2004; Talmadge JE, 2007). These cells are preferentially localizedin the poorly vascularized regions of tumors (Lewis CE, et al.; 2006; Leek RD, 2002) where the environment promotes their metabolic adaptation to hypoxia through the activation of hypoxia-inducible factor 1 (HIF-1) and 2 (HIF-2) (Leek RD, 2002).

M2 cells, producing also IL-10 and TGF-β, contribute to general suppression of anti-tumour activities. Moreover, the cross-talk between the macrophages and the neoplastic cells ensures the continuous process ofdeposition and remodelingof extracellular matrix, which facilitates tumor growth and invasion of the surrounding tissue.

Furthermore, the M2 macrophages enhance the tumor initiation andthe metastasis progression(Liu C, et al., 2013), while the M1infiltration is associated with the tumor regression and a favorable outcome of survival (Cook RS, et al., 2013; Helm O, et al., 2014; Maeda R, et al., 2014).

Recentlyhas been demonstrated that the overexpression of MERTK in epithelial cancer cells results in increased motility and chemoresistance with a strong gain of function and ability in efferocytosis. In the same model, the overexpression of MERTK induces Programmed death-ligand 1 (PD-L1) and PD-L2 expression, a known blockade of immune checkpoint in cancer, which could alter the immune balance for tolerance and progression (Khanh-Quynh N, et al., 2014).

Since the efferocytosis MERTK-mediated, tends to release factors able to promote tissue remodeling and immune system suppression, it is possible that cancer cells adopt "MERTK enhanced efferocytosis" as a strategy for the tumor tolerance ((Khanh-Quynh N, et al., 2014).

1.4 LIVER FIBROSIS

Liver fibrosis is a main feature of the chronic liver injury and is characterized by the conversion of the "normal low-density basement membrane-like matrix" to the "high-density interstitial type matrix", containing fibrils that form collagen. The extensive liver fibrosis derives from the deposition and the accumulation of type I collagen and is recognized as cirrhosis (Dan L, et al.; 1999).

Liver fibrosis is reversible, whereas cirrhosis is generally irreversible. The clinical complications of cirrhosis include the portal hypertension and the chronic liver failure.

Normal liver contains epithelial cells (hepatocytes), tissue macrophages (Kupper Cells) and perivascular mesenchymal cells (HSCs). HSCs are organized within the sinusoid, with the sub-endothelial space of Disse that separates the epithelium from the sinusoidal endothelium. In normal liver this space contains a "basement membrane-like matrix" (*Fig.5*) (Scott L, Friedman, 200).

When liver becomes fibrotic, the composition of hepatic ECM is modified, both qualitatively and quantitatively. The collagen and non collagenous components increases 3-5 fold, accompanied by the shift in the type of ECM in the sub-endothelial space, that becomes an "interstitial type matrix containing collagens" (*Fig.5*) (Scott L. Friedman, 200).

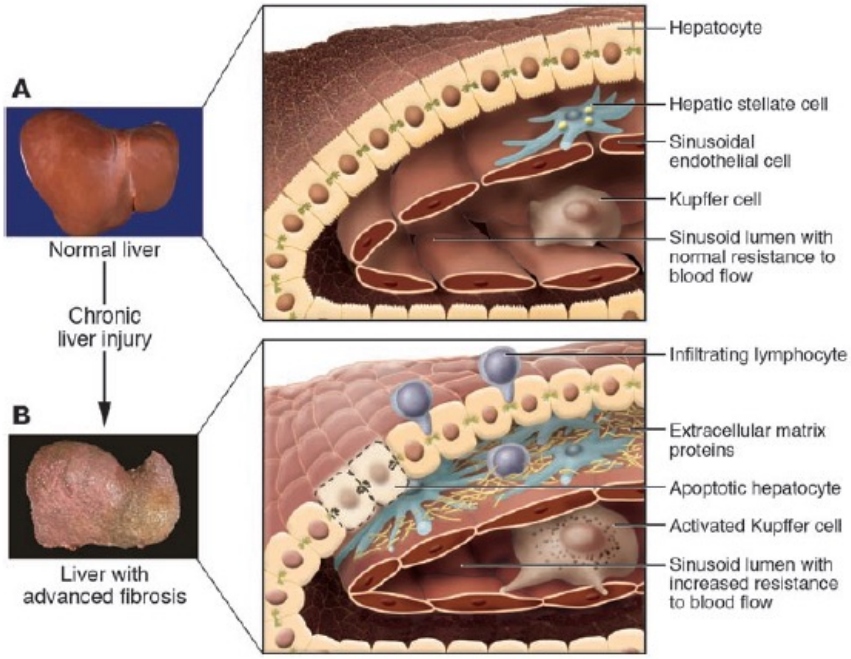

Figure 5.Phenotypic features of the sinusoidal endothelium in the normal liver and during the liver injury.

The HSCs are the key fibrogenic cells in injured liver and have been identified as important effectors in the hepatic inflammation (Bataller R. et al.; 2005).

Quiescent HSCs are primary cells in the body responsible for the storage of the vitamin A (Bataller R, et al., 2004; Bataller R. et al.; 2005). Upon activation by liver injury, the quiescent HSCs trans-differentiate into myofibroblasts, which produce inflammatory cytokines and secrete collagen types I and III, the main proteinsof the matrix, responsible for the development of liver fibrosis and cirrhosis (Friedman S.L. 2000).

Reactive oxygen species (ROS), EIIIA isoform of fibronectin, produced by endothelial cells, and other paracrine stimuli generated from platelets (PDGF, TGF-β and EGF) are implicated in the initiation phase of the HSCs activation (Eng and Friedman, 2000). Additionally, during this phase, the endothelial cells convert the latent TGF-β to the active fibrogenic form, through the activation of plasmin (Friedman S.L. et al.; 1999).

TGF-β, seems to play a dominant role in fibrogenesis, participating in the multiple phases of the HSCs activation (Blobe GC, et al.; 2000; Dooley S, et al.; 2001; George J, et al.; 1999). This cytokine is a strong fibrogenic signal (Friedman SL, 2000; Friedman SL, 1999) and induces collagen I, a major constituent of the cirrhotic scar (Canbay A, et al.; 2003).

The hepatocytes also promote the activation of HSCs by producing lipid peroxidesthat increase the proliferation of HSCs and the synthesis of the collagen I (Gressner AM, 1995).

The hepatocytes represent the primary source of the apoptotic bodies, characteristic of the liver injury (Rust C. et al., 2000). The apoptotic cells are subsequently cleared not only by

professional phagocytes but also by HSCs (Platt et al.; 1998). When the HSCs engulfs the apoptotic bodies, they are activated and generate directly fibrogenicstimuli.

After the initiation, the activated HSCs, undergo a series of the phenotypic changes, that lead to accumulation of extracellular matrix.

The perpetuation of HSCs activation involves key phenotypic responses, mediated by the cytokines and byECM remodeling (Friedman S.L. et al.; 1999).

The enhanced cytokine response occurs through multiple mechanisms; among these, increased expression of cell membrane receptors and enhanced signaling are specially important (Pinzani M. et al.; 1998).In particular,RTKs, which mediate many of the responses of HSCs to cytokines, are up-regulated during liver injury (Ankoma-Sey V, et al:, 1998; Ankoma-Sey V, et al:,1998).

During this phase, the continuous ECM remodeling, underlies all cellular responses that characterize the progressive liver injury. The low density subendothelial matrix is progressively replaced by a rich in collagen fibril form (Scott L et al.; 2000) which accelerates the activation of HSCs. These effects are mediated not only through interaction with the integrins, the classic receptors of ECM, but also through binding at least to one RTK.

The phenotypic response of HSCs, after liver injury, includes: proliferation, contractility, activation of the fibrogenesis, chemotaxis, matrix degradation, loss of retinoid, release of cytokines and leucocytes chemoattraction.

The HSCs proliferation is induced by platelet-derivated growth factor (PDGF) and is associated with both increased of the autocrine PDGF production and up-regulation of PDGF receptor (PDGFR) (Pinzani M, et al.; 1998). The activated PDGFR recruits the signaling molecule Ras and actives ETK/mitogen-activated protein kinase pathway (Marra F, et al.; 1997).

The contractility, property of activated HSCs, is induced by the endothelin-1 (ET-1), which is in part derived by an autocrine mechanism and represents an important mechanism for the increased of the portal resistance during liver injury (Rochey DC, 1997; Rockey DC, et al.; 1998).

The fibrogenesis is characterized by ECM production from HSCs and is mediated by the TGF-β. This cytokine has an important role in perpetuating rather than in initiating the activation of the HSCs (Hellerbrand C, et al.; 1999).

The chemotaxis, the directed migration of the HSCs activated,increasestheir accumulation in the areas damaged.The PDGF and the monocyte chemotactic protein-1 (MCP-1) have been identified as the chemoattractants for the HSCs activated but not for those quiescent (Marra F, et al.; 1997; Marra F, et al.; 1999).

The matrix degradation is a result of the change in matrix proteases activity, that leads to remodeling of the ECM in liver injury, acceleratingtheHSCs activation. In particular, the HSCs are a key source of the matrix metalloproteinase-2 (MMP-2) and the stromelysin/ MMP3. These cells activated up-regulate also the tissue inhibitor of metalloproteinase -1 and -2 (TIMP-1 and -2)

and inhibit the activity of interstitial collagenases, favoring the scaraccumulation (Arthur MJ, 1995).

The loss of retinoid is a feature of the activation of the HSCs, but remains unknown whether the loss of the intracellular vitamin A is required to activate the HSCs and whether the retinoids might accelerate or prevent the their activation.

Finally, the cytokines release and the leucocyte chemoattraction are critical for the perpetuation of HSCs activation. All features of HSCs activation can be attributed to autocrine cytokines (Friedman SL, 1999) and the liver ECM represent an important reservoir of growth factors. The HSCs can also amplify the inflammation through the release of chemoattractants for neutrophil and monocyte (e.g. MCP-1) (Marra F, etal.; 1998).

The chronic hepatic inflammation, that leads to fibrosis, occurs in response to a variety of insults that include viral hepatitis, alcohol and drugs consumption, non alcoholic steatohepatitis and metabolic disease due to overload of iron or copper. The complications of fibrosis and its progression includes cirrhosis and HCC.

1.4.1 PROGRESSION OF LIVER DISEASE IN CHRONIC HEPATITIS C

Chronic Hepatitis C (CHC) is the most common cause of chronic liver disease and cirrhosis and the most common indication for liver transplantation in the United States (U.S.), Australia, and most of Europe (Wasley A, et al., 2000; Alter MJ, et al., 1997; Charlton M, et al. 2001). The prevalence of CHC is estimated at 3% (World Health Organization. 2000) and only a minority of those infected spontaneously clears the virus.

Most of the HCV infections (70-80%) persist and about 30% of these individuals develop chronic liver disease (Micallef JM, et al., 2006) including cirrhosis (Seef LB, 2002) which is a prerequisite for hepatocellular carcinoma (HCC) development (Hoofnagle JH, et al., 2002; Thomas DL, et al., 2005). Factors related both to virus and to host have been associated with the differences in HCV clearance or persistence.

Current standard of care therapy for CHC is the polyethylene glycol (PEG)ylated interferon α and ribavirin (PEG-IFN-α/RBV) (Di Bisceglie, AM, et al., 2002).

The Hepatitis C Virus (HCV) is an RNA-virus that belongs to Flaviviridaefamily. Its genome consists of 10 kb and includes the 5'untranslated region, four structural proteins (core, envelope protein 1, E1 and E2) , six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), and a 3'-untranslated region. This genome is initially translated into one single polyprotein and subsequently cleaved into the functional proteins (Moradppour D, et al., 2004).

The HCV enters into hepatocytes via interactions between the envelope viral proteins (E1 and E2) and the host cell receptors, such as CD81, scavenger receptor class B1 and the tight junction proteins (Liu S, et al., 2009; Pileri P, et al., 1998; Scarselli E, et al., 2002). Whereas HCV replicates in the cytoplasm of hepatocytes, it is not directly cytopathic, but he persistent infection and the rapid production of virus activates a vigorous T-cell immune response. In particular, the destruction of the infected hepatocytes and the viral clearance are mediated by distinct T-cell populations (Neurmann-Haefelin C, et al., 2005).

When $CD8^+$ cells are activated, at first, fail to produce interferon-γ (IFN-γ); so this phase is characterized by a small reduction of viremia. Subsequently, the $CD8^+$T cells switched to produce IFN γ and, in the same time, the number of $CD4^+$T cells increased and the viremia decreased of 5-log(Herz K, et al., 2007).

This evidences suggest that hepatocytes destruction, disease pathogenesis and viral clearance are mediated by different effector mechanisms (Herz K, et al., 2007).

Furthermore the death receptors, apoptosismediators, play a critical role in the liver injury associated to HCV. In particular, CD95/CD95L system seems to play the most important pathogenic role (Hayashi N, et al., 1999). The up-regulation of CD95 in hepatocytes (Hiramatsu

N, et al., 1994), as well as the induction of CD95-L expression in T lymphocytes, (Mita E, et al., 1994) have been correlated with the severity of the inflammation.

The hepatocytes destruction and their apoptosis are prominent features of the HCV infection. The apoptosis acts together with the immune response to clear host infected cells and plays an important role in cells elimination that undergo uncontrolled cellular proliferation and in preventing viral replication (Nerz K, et al., 2007).

In addition to the activation of the inflammatory response, HCV can modulate the metabolism of the infected hepatocytes. The mechanism by which virus acts to induce derangement of the lipid metabolism involves the core, NS3 and NS5A viral proteins (Lerat H, et al., 2002; Perlemuter G, et al., 2002; Moriya K, et al., 1997). These viral proteins are also able to modulate the hepatocyte apoptosis favoring hepatocarcinogenesis (Gong G, et al., 2001; Arima N, et al., 2001).

The core protein is associated with the lipid droplets in the cytoplasm of hepatocyte but it is also present in the mitochondria and the nuclei (Moriya K, et al., 1998; Moriya K, et al., 1997) and it acts differently depending on the presence or absence of inflammatory condition.

The core protein activity, under inflammation, contribute to oxidative stress condition that alters the scavenger system impairing the elimination of damaged cells (Moriya K, et al., 2001); so the amplification of inflammatory response and the progression of chronic disease is observed.

Alternately, the core protein is able to increase the production of oxidative stress with consequent over-production of ROS in absence of the inflammatory conditions (Moriya K, et al., 2001; Moriya K, et al., 2001). This event results in mitochondrial DNA delection indicating genetic damage.

HCV can also induce liver fibrosis independently from inflammation: it has been demonstrated that uptake of HCV by HSC, via specific receptors, could play a role in fibrogenesis through the modulation of profibrogenic signaling.

It was been demonstrated that, in the hepatocytes infected, the induction of ROS by viral proteins NS3 and NS5B, up-regulates TGF-β production (Gong G, et al., 2001; Arima N, et al., 2001). Indeed, these viral proteins interact with the mitochondria and induce lipid accumulation and degradation, with consequent compartimentalizationand metabolism derangement, resulting in the production of ROS (De Bleser PJ, et al., 1999). ROS induce TGF-β production (Garcia-Trevijano ER, et al., 1999) andthe same TGF-β amplifies theirproduction (Moriya K, et al., 2001; De Bleser PJ, et al., 1999; Pociask DA, et al., 2004; Scuppan D, et al., 2003).

TGFβ acts as a potent inducer of ECMaccumulation, activating the quiescent HSCs to myofibroblasts (Cassiman D, et al., 2002). These cells up-regulate the synthesis of interstitial collagens and down-regulate the MMP (Gressner AM, et al., 2002).

In addition, viral proteins stimulate the synthesis of mediators profibrogenic and induce the activation of p38, MAPK, JNK, ERK and NF-kappa B pathway (Lin W, etal., 2010).

In particular, NS5A is involved in PI3K/AKT and β-catenin/WNT activation pathways and in the escape from apoptosis through caspase -3 inhibition (Street A, et al., 2005).

The core protein is able to suppress SOCS-1, a tumor suppressor gene, induce the generation of ROS and transactivate MAPK and AP1 pathways. The activation of the MAPK pathway, in combination with the oxidative stress, contributes to give an advantage to hepatocytes for cell proliferation and hepatocarcinogenesis (Koike K. 2006).

The core protein inhibits also the immune responses through NF-kappa B activation (Joo M, et al., 2005), whereas the activation of JNK pathway generates the inflammatory hepatic microenviroment that supports HCC development (Hui L, et al., 2008).

The viral proteins subvert also the signaling of the innate immune, through the inhibition of natural killer cells and the impairment of the antigen-presenting cells (Li XD, et al., 2005; Tacke RS, et al., 2011).

Thus, the core protein and other viral proteins are capable to induce the overproduction of oxidative stress in the absence of inflammation and can contribute also to the hepatocarcinogenesis during the HCV infection.

In CHC another source of the over-production of oxidative stress is the hepatic steatosis (Moriya K, et al., 2001; Shintani Y, et al., 2004; Koike K, et al., 2005).

The mechanism of the HCV associated steatosis shares similarities with that involved in NAFLD and requires “two hit” for its progression (Day CP. 2002; Day CP, et al.,1998).

The alteration of the double membrane structure within mitochondria, mediated from core protein, causes an impairment of the lipid oxidation and acts as the “first hit” for the development of steatosis. Indeed, the core protein, that has as target the microsomal triglyceride transfer protein (MTP), interferes with the assembly and the secretion of the apolipoprotein (apo) B, containing very low density lipoprotein (VLDL) (Perlemuter G, et al., 2002).More recently, it was been shown that the core protein may induces the oxidative stress directly acting on the mitochondrial electron transport (Okuda M, et al., 2002).

The activation of antiviral inflammatory responses represents the “second hit” in CHC for the progression from steatosis to fibrosis. (Moriya k, et al., 1998).

The antiviral inflammatory responses represents a source of free radicals, as well as of pro-inflammatory and profibrotic cytokines. In support of this, it was been demonstrated that the degree of sinusoidal fibrosis, in additional to the steatosis, correlates with the severity of both portal and lobular inflammation (Clouston AD, et al., 2001).

Thus, the induction of oxidative stress, mediated by HCV, and its role in fibrosis, represents the major mechanism for the progression of the hepatic disease and for the

development of HCC, driven from cirrhosis, in patients with CHC (Yoshida H, et al., 1999; Fattovich G, et al., 2004).

1.4.2 PROGRESSION OF LIVER DISEASE IN CHRONIC HEPATITIS B

Chronic infection with hepatitis B virus (HBV) affects about 400 million people and is estimated that worldwide over 200,000 and 300,000 chronic HBV carriers die each year respectively from cirrhosis and HCC (EASL 2003; Perez JF, et al., 2006).

HBV, an encapsulated virus, belongs to hepadnaviridae family and show preferential tropism for hepatocytes, where replicates through an RNA intermediate. This virus has a circular partially double stranded DNA genome of approximately 3.2 kb pairs (Summers J, et al., 1982).

The genome contains four open reading frames (ORFs) that cover the entire genome: pre-S/S ORF encodes three viral surface proteins; pre-C/C ORF encodes the antigen "e" (HBeAg) and the antigen "core" (HBcAg); P ORF encodes the terminal protein (TP) and the viral polymerase, that has DNA polymerase, reverse transcriptase and of RNaseH activities. The X gene encodes a small protein essential for virus replication. Finally, HBSP is a viral protein that is encoded by a spliced viral transcript but its function is unknown (Soussan P, et al., 2000).

The natural history of chronic infection by virus B (CHB) involves four key phases, that are defined based upon the combination of the serum markers (Hadziyannis SJ. 2011).

When HBV infects host cells, estabilishes an acute infection that can become chronic depending on the age and the modes of transmission.

The acute infection becomes chronic if HBV, that is infecting, is wild type in precore region and is able to produce HBeAg. However the expression of HBe protein requires the host immune tolerance against HBV(Cote PJ, et al.,2000; Hadziyannis SJ, et al., 2001).

Once HBV infection has become chronic, the subsequent course consists of four variable duration phases depending on a balance between viral parameters and immune host response (Ganem D, et al., 2004).

The first two phases are associated with HBeAg seropositivity, while the other two are characterized by the clearance of HBeAg and the development of anti-HBe immunity (Milich DR. 1989; Milich DR. 1991).

However, a subgroup of patients that undergo seroconversion to anti-HBe antibodies and becomes HBeAg-negative, are characterized by periodic reactivation. This event is associated with fluctuating levels of alanine aminotransferase (ALT), HBV DNA positivity and an active hepatitis with variable fibrosisdegree. By contrast, loss of HBsAg during the second phase is associated with a favorable prognosis, although the incidence of HCC remains higher respect to not infected population (Paterlini P, et al., 1991).

Hepatocytes apoptosis is feature of HBV infection and cytotoxic lymphocytes T (CTLs) exert an important role in the pathogenesis of disease. Indeed, CTLs activate simultaneously the death receptor CD95 and the perforin pathways, the principal responsible for the killing and damagecellular, in absence of HBsAg retention.

In contrast, the IFNγ pathways are primarily responsible for killing of hepatocytes with HBsAg retention independently of presence or absence of CD95 receptor or perforin signaling (Herzer K et al., 2007).

The most of HCC develop in cirrhotic livers, whereas a significant fraction of liver cancers, HBV-related, occur in absence of liver cirrhosis. The low rate of cirrhosis can be explained with the ability of HBV to integrate into the host genome and to play, potentially, a direct carcinogenic activity.

The integration of viral DNA causes the alteration of human genome, leading to mutations of genes implicate in cell proliferation, migration, differentiation and survival (KM; Sze, et al., 2013;WK, Sung et al., 2012; Y, Tao et al., 2011). Indeed, it was been shown that HCC, induced by HBV, displays also higher rates of chromosomal alterations (Marchio A, et al., 2000) and implies also a complex interaction between viral and host factors.

HBsAg is able to compromise hepatocellular function (Chisari FV, et al., 1995) through its accumulation in the endoplasmic reticulum (ER) and the formation of hepatocytes to "ground-glass"(Pasquinelli C, et AL., 1992).

HBsAg can also play a direct role in HCC development: accumulating in ER, can induce oxidative stress, which cause DNA damage and alterations of pathways related to cell death mechanisms, survival, proliferation, invasion, and apoptosis(Pasquinelli C, et AL., 1992).

HBx is a viral protein present at high concentrations in the cytoplasm and at low concentrations in the nucleus. At cytoplasmatic level activate apoptosis through localizing in mitochondria, or stimulate HBV replication through localizing in the nucleus (Keasler VV, et al., 2009). This protein can also induce apoptosis by promoting ROS generation, activating caspasi 8 and removing the mitochondrial membrane potential. In addition, HBx induce apoptosis up-regulating Fas-Ligand (Fas-L) (RX, Tang, et al., 2012) and interacting with Bcl2/CED-9 signaling (X, Geng, et al., 2012).

The chronic inflammation status plays an important role in HCC development: cycles of cellular inflammation, that induce apoptosis, and regeneration of the hepatocytes, increase the risk of HCC. Cytokines, such as interleukin 6 (IL-6) TNF-α and TGF-β, play an important role in this contest.

In the early stage of disease, TGF-β promotes cirrhosis progression by accelerating the deposition of the extracellular matrix (Matsuzaki K, et al., 2009), whereas, when cancer occurs, its persistent up-regulation accelerates the neoplastic growth (Rossmanith W, et al., 2001).

Finally, the epigenetic changes contribute to hepatocarcinogenesis through the interaction of the HBx protein with molecular factors that mediate the phosphorylation or the methylation of the cellulartargets (E, Khattan, et al., 2012; Y, Huang, et al., 2013). In particular, HBx induce hypermethylation of tumor suppression genes and interact with the histone acetyltransferase or methyltransferase (Y, Tian, et al., 2013).

1.4.3 PROGRESSION OF LIVER DISEASE IN NON ALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic Fatty Liver Disease (NAFLD) is characterized by excessive accumulation of triglyceride within the liver and comprises a large spectrum of disease, including hepatic steatosis and non-alcoholic steatohepatitis (NASH), which can progress to the cirrhosis and its complications, like the hepatic failure and the HCC (Ong JP, et al., 2007).

It was been proposed the "two hit hypothesis" to explain the molecular mechanism for the disease progression from NAFLD to NASH. According with this hypothesis, the "first hit" is the accumulation of lipids into the hepatocytes, that results in the simple steatosis; whereas the "second hit" is caused by the oxidative stress and subsequent necro-inflammation, that leads to the NASH (Day CP, et al., 1998; Day CP, et al., 1998). However, kupffer cells and HSCs contribute to progression from the simple steatosis to the NASH (De Minicis S, et al., 2011).

The free fatty acids (FFAs) play a crucial role in the direct promotion of liver injury. Indeed, insulin resistance, associated with obesity, metabolic syndrome and diabetes, leads to increased release of the FFAs from the adipocytes and their increased influx in the liver, where FFAs undergo β-oxidation or are esterified with glycerol to form the triglycerides. The accumulation of triglycerides in the hepatocytes increases the oxidative stress that activates the inflammatory pathways (Feldestein AE, et al., 2004). Additionally, the oxidative stress inhibits the replication of mature hepatocytes, resulting in the inadequate expansion of the hepatic progenitor cell population, (Roskams T, et al., 2003) and induces an excessive death of the hepatocytes (Jou J, et al., 2008).

The cytotoxic products from lipid peroxidation, that impaire the cellular functions of the hepatocytes, activate also apoptotic mechanisms that, if on one hand represent an essential process to resolvethe inflammation, on the other hand play a role in fibrogenesis by modulating the HSCs activation and collagen expression (Ikejima K, et al., 2007; Ahima RS, et al., 2008).

Moreover, NASH has been proposed as a probable cause of the idiopathic or cryptic cirrhosis (CC) (Bugianesi E, et al., 2002; Caldwell SH, et al., 1999; Poonawala A, et al., 2000) that can progress, although rarelyandslowly, to HCC (Bugianesi E, et al., 2002; Marrero JA, et al., 2002; Regimbeau JM, et al., 2004).

The exact mechanism that regulates the development of HCC in NASH patients is not clear. Obesity and diabetes are the risk factors for the development of NASH and CC and they have been implicated in the hepatocarcinogenesis (Bugianesi E. 2007).

Different factors are implicated in the progression of disease from NASH to the HCC, as the insulin resistence, the ROS production, the oxidative stress and the inflammation.

The insulin resistence and the resulting hyperinsulinemia up-regulates the production of the nsulin-like growth factor-1 (IGF-1) that stimulates the growth through cellular proliferation and apoptosisinhibition of the hepatocytes (Page JM, et al., 2009).

ROS production, that is increased in the mitochondria of the steatotic hepatocytes, is implicated in the hyperplasia (Bugianesi E, 2007; Yang S, et al., 2000) that generally precedes the tumorigenesis for many years (Buginesi E, 2007).

Cancer developmentis supported by oxidative stress through the induction of tumorigenic mutations e.g. in the gene p53 (Hu W, et al., 2002), and in the patients with NASH can also be mediated by increased inflammatory cytokines release and NF-kB activation (Luedde T, et al., 2007). NF-kB activates a chronic cycle of injury, which goes from cellular death to regeneration, and that contributes to hepatocarcinogenesis (Luedde T, et al., 2007).

Finally, c-Jun amino-terminal kinase 1 (JNK1), activated by TNF-α and ROS, was recently been linked to NASH and HCC by increasing of hepatic inflammation and apoptosis (Puri P, et al., 2008).

1.4.4 HEPATOCELLULAR CARCINOMA (HCC)

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third cause of cancer mortality (Shariff MI, et al., 2009).

It is related to the inflammation and liver fibrosis status (Seitz HK, et al., 2006) but, specifically, the chronic inflammatory state appears to be necessary for its initiation and development.

Several studies reported that viral chronic infections are the major risk factors for the development of liver tumor but other risk factors, including alcoholic cirrhosis (1,7%) (Fattovich G, et al., 2004), nonalcoholic steatohepatitis (2,6%) (Ascha MS, et al., 2010) and hemochromatosis (Kris VK, et al., 2004) are known.

The development and progression of HCC is a multistep process. A chronic insult, e.g. HCV, HBV or free fatty acids, induces liver injury through ROS production, ER stress, necrosis and hepatocytes death.

In particular, HCV acts through the activation of immune inflammatory response that promotes the neoplastic transformation.

HBV, instead, can integrate into the host genome and promote directly the hepatocarcinogenesis through a sustained inflammatory damage, regeneration of hepatocytes, alteration of DNA repair mechanisms, direct oncogenic transformation and transactivation of several viral oncoproteins as HBx.

The hepatic response involves the HSCsand macrophages activation: the first, produce ECM components and growth factors, while the second promote the migration of endothelial cells, angiogenesis and fibrosis. These processes, in the context of inflammation and oxidative damage to DNA, favoring the mutations accumulation and epigenetic aberrations in the pre-neoplastic hepatocytes and in the liver stem cells, promote the development of dysplastic nodules and their malignant transformation (Seitz HK, et al., 2006).

The stromal liver components, such as the angiogenic cells, the endothelial cell, the HSCs, the carcinoma-associated fibroblastic cells (CAFs) and the immune cells, contribute to development of cancer in several manners: sustaining the proliferative signaling, evading the growth suppressors, resisting the cell death, enabling the replicative immortality, inducing the angiogenesis, activating the invasion and the metastasis, reprogramming the energy metabolism and evading the immune response (Hanahan D, et al., 2012).

The angiogenesis plays an important role in early stages of cancer development (Zhu AX, et al., 2011): the tumor massgrowth creates an environment deprived of nutrient and oxygen and induces the activation and the proliferation of endothelial cells (ECs) to generate new vessels from preexisting ones (North S, et al., 2005).

The ECs liberate enzymes to disrupt the membrane basement and migrate to their final location where they form new vessels together with the ECM. In particular, ECM is involved in vessel lumen formation, in tubulogenesis and deposition of a supportive membrane basement (Coulon S, et al., 2011).

The ECM, normally, is essential to support the liver architecture (Lu P, et al., 2012) but, in the hepatic disease, the activity of remodeling enzymes is deregulated; this leads to a fibrotic microenvironment, characterized by increased of stiffness and by abundance of growth factors that contribute to tumorigenesis (Lu P, et al., 2012) and directly affect the epithelial cells with consequent cellular transformation and metastasis.

Inflammation predisposes at the beginning of cancer and several inflammatory mediators have been implicated. In particular, the TH2 inflammatory response is associated with HCC phenotype more aggressive and metastatic (Budhu A, et al., 2006; Budhu A et al., 2006).

High levels of IL-6 and IL-22, that are produced by kuppfer cells in response to hepatocyte damage, have been detected in tumoral microenvironment. These cytokines lead to tumor growth, apoptosis inhibition, and metastasis promotion through the Signal Transducer and Activator of Transcription 3 (STAT3) activation (Tilg H, et al., 1992; Jiang R, et al., 2011). IL-6 exerts also its oncogenic activity by triggering downstream ERK pathways, which controls target genes involved in both cell proliferation and survival (Maeda S, et al., 2005).

The TNF-α is strongly involved in HCC pathogenesis by promoting invasion, angiogenesis and metastasis (Lewis CE, et al., 2006) and by activating the phosphorylation of p38, MAPK, ERK and by IL-8 production (Wang Y, et al., 2012). TNF-α stimulates also the activation of CAFs, dominants cells in tumoral inflammatory environment.

The IL-10 is the most important immunosuppressive cytokine that, together with TNF-α, reduces the $CD8^+$ cells activity and supports the tumor immune escape (Kuang DM, et al., 2009; Ke W, et al., 2009). It is also involved in the induction of Forkhead Box P3 ($FOXP3^+$) that regulates T cell differentiation. $FOXP3^+$ suppresses the activity of the effector T cells and is associated with aggressive HCC (Zhou J, et al., 2009).

The chemokines are critical in the chemotactic and trafficking cell into and out from the tumor microenvironment and have been implicated in immune system evasion, angiogenesis, invasion and dissemination (Roussos ET, et al., 2011).

NF-kB, which plays a key role in HCC development, is activated in epithelial cells and macrophages from pro-inflammatory stimuli, such as hepatitis virus (Kim HR, et al., 2010;) and free fatty acids (Shi H, et al., 2006). Activated NF-kB induces the growth and the survival of malignant hepatocytes through the release of the inflammatory cytokines, such as IL-1α.

The immune system plays an crucial role in the development of HCC and the tumor-associated macrophages (TAMs) are a major component of leukocyte infiltrate.

TAMs establish a crosstalk between tumor and stromal cells (Mantovani A, et al., 2011) and their recruitment leads to secretion of soluble mediators, which support the tumor cell survival, proliferation and dissemination.

In the tumoral microenvironment, TAMs are mainly polarized in M2 phenotype and PGE2, TGF-β, IL-6 and IL-10 are the factors that promote this polarization (Mantovani A, et al., 2005; Chomarat P, et al., 2005).

In addition these cells produce immunosuppressive cytokines like IL-10 and TGF-β and molecules, such as EGF, VEGF, PDGF that directly affect tumor cell proliferation and connective tissue dissolution (Lewis CE, et al., 1995).

The M2polarized macrophage functions, expressed during the development of tumor have an important impact on its progression (Mantovani A, et al., 2002). The major regulators of their transcriptional programs are NF-kB, STAT-3 and p50, that concur to establish the permissive conditions for tumor progression (Sica a, et al., 2007).

In addition, TAMs up-regulate the expression of CXCL8 receptor and accumulate preferentially in the poorly vascularized regions of the tumor. These regions, where the TAMs activate the pro-angiogenic program with consequent expression of VEGF through a mechanism mediated by HIF are characterized by low oxygen tension (Talks KL, et al., 2000; Pollard JW, 2004; Knowles HJ, et al., 2007).

TAMs produce also the enzymes and the inhibitors involved in the extracellular matrix digestion, and are able to release several matrix metalloproteases (MMP2, MMP9) and activators of MMPs, favoring the tumor invasion.

Finally, TAMs suppress the adaptative anti-tumor immune responses through the release of IL-10, prostaglandins, TGB-β and indoleamine dioxigenase (IDO) metabolites (Mantovani A, et al., 2002). In addition, various cytokines, e.g. IL-6 and IL-10, present in tumor environment contribute to promotes the differentiation of monocytes into mature macrophages and blocks their differentiation into dendritic cells (DC) (Allavena P, et al., 2000).

1.5 OUTLINE OF THESIS

Mer tyrosine kinase (MerTK) is a major macrophage receptor involved in the clearance of apoptotic cells and is expressed principally in a subpopulation of macrophages defined M2. The M2 cells, together with the M1, belong to Tumor Associated Macrophages (TAMs) and are one of the most important players in inflammation and cancer, being also a major source of cytokines (Mantovani A, et al., 2008). Most of the TAMs that are involved in promoting the tumor, angiogenesis and tissue remodeling have a M2 phenotype (Sica A, et al., 2008).

MerTK is encoded from MERTK gene, located on the chromosome 2 and is involved mainly in efferocytosis, but can also stimulate the fibrogenesis acting directly on the HSCs(Fadok VA, et al., 1998; Platt N, et al., 1998).

Genome wide Association (GWA) study reported that the single nucleotide polymorphism (SNP) of MERTK (rs4374383) is associated with the risk of developing fibrosis in patients with CHC(Patin E. et al., 2012). It seems that the state polymorphic of the MERTK gene (rs4374383 G> A) may play a key role not only in the control of apoptosis, in the epithelial-mesenchymal transition of the HSCs and in the immune response, but also in the mechanisms involved in fibrosis progression and carcinogenesis.

On the basis of this results, we investigated if the MERTK gene and the variant rs4374383 (G> A) can influence the risk of progression of the liver disease, in patients with chronic infection from the hepatitis viruses (C and B) or with nonalcoholic steatohepatitis (NASH).

Specifically, in **chapter 2,** we evaluated if the rs4374383 SNP influences the risk of liver decompensation (LD) and HCC in patients with HCV cirrhosis,treated with Peg-interferon alfa-2b and ribavirin.

In **chapter 3** we assessed the potential association between the rs 4374383 SNP in the MERTK gene and the risk of the development of HCC in a cohort of HBV patients with active HBV replication and in a sub cohort of them who achieved a virological suppression on long term NUCs treatment.

Finally, in **chapter 4,** we evaluated if thers4374383 SNP of the MERTK gene was associated with the severity of liver fibrosis and histological features in two combined cohorts of 533 patients with NAFLD. We also investigated if the MERTK gene is expressed in human HSC and in murine models of fibrogenesis.

CHAPTER 2

A rs4374383 Single Nucleotide Polymorphisms of MERTK gene is linked to a higher likelihood of hepatocellular carcinoma in patients with HCV cirrhosis

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Abstracts of the Italian Association for the Study of the Liver (AISF) Monothematic Conference 2013 "Personalized care in Hepatology" Pisa, 17–19 October 2013

RS4374383 SINGLE NUCLEOTIDE POLYMORPHISM OF MERTK GENE INFLUENCES THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA (HCC) IN PATIENTS WITH HCV CIRRHOSIS

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Background and aims: MERTK gene, located in chromosome 2, encode factors involved in phagocytosis of apoptotic cells by macrophages. We evaluated if rs4374383 SNP influenced the risk of liver decompensation (LD) and hepatocellular carcinoma (HCC) in patients with HCV cirrhosis

Methods: We evaluated a prospective cohort of patients with compensated HCV cirrhosis treated with Peg-interferon and ribavirin. Genotyping for rs4374383 SNP was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). All patients were screened for esophageal varices (EV) and underwent to surveillance for HCC every six months.

Results: Among 349 patients (mean age 58 ± 8.6 years, 61.2% men, 85% genotype 1); 16.9% had AA genotype, 46.4% GA and 36.9% GG of rs4374383 SNP; 50.7% had EV. Eighty-seven patients (24.9%) achieved a Sustained Virological Response (SVR). During the follow-up (median 77 months; range 12-145) LD was observed in 6 (6.8%) SVR and in 71 (27.1%) no SVR ($p < 0.001$), while 6 (6.8%) with SVR and 66 (25.2%) without SVR developed HCC ($p < 0.001$). By multivariate analysis presence of EV (HR 3.11; 95%CI: 1.69–5.75; $p < 0.001$), platelet count (HR 0.99; 95%CI: 0.98–0.99; $p = 0.001$), albumin (HR: 3.11; 95%CI: 0.19–0.54; $p < 0.001$), and absence of SVR (HR: 4.04; 95%CI: 1.63–10.05; $p = 0.003$) were independently associated to LD. The variables independently associated to development of HCC were age (HR 1.04; 95%CI: 1.01–1.07; $p = 0.045$), GGT (HR 1.14; 95%CI: 1.20–1.37, $p = 0.008$), absence of SVR (HR 3.31; 95%CI: 1.43–7.68; $p = 0.005$) and the AA genotype of rs4374383 SNP (HR 2.67; 95% CI: 1.36–5.23; $p = 0.004$). The risk to developed HH was of 1.04, 2.43, 4.05 and 7.17 per 100 persons/years in patients with SVR and no responder to therapy with genotype GG, GA and AA of rs4374383 SNP, respectively.

Conclusion: The AA allele of rs4374383 SNP of MERTK gene is associated with a high risk to developing HCC in HCV cirrhosis.

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NORMOCALORIC LOW CHOLESTEROL DIET MODULATES TH17/TREG BALANCE IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS INFECTION

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Background & aims: Hepatitis C virus infection is characterized by its association with several autoimmune disorders. Chronic hepatitis C (CHC) patients display increased amount of T helper 17 (Th17) cells. Th17 are involved in both liver inflammation and autoimmunity. In CHC patients, serum levels of oxysterols, the specific ligands of Liver X Receptors (LXRs) and non-enzymatic cholesterol oxo-derivatives molecules, are higher than in non-alcoholic fatty liver disease (NAFLD) patients. Interestingly, LXR activity influences the differentiation of Th17 cells; besides Hepatitis C virus direct interferes with the activity of LXR/RXR heterodimer. We evaluated whether a Normocaloric Low Cholesterol Diet (NLCD) may modulate peripheralTh17/Treg balance, in a cohort of CHC patients, taking NAFLD/NASH patients as control group.

Methods: Thirty CHC and thirty NAFLD/NASH patients were treated with a NLCD for 30 days. Before and after NLCD,

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CHAPTER 3

The risk of hepatocellular carcinoma in HBV cirrhosis is affected by polymorphisms of the MERTK gene

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Abstracts of the A.I.S.F. Monothematic Conference 2014: Poster session

1

MYCOPHENOALATEMOFETIL MONOTHERAPY IN LIVER TRANSPLANTATION

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Background: Mycophenoalatemofetil (MMF) is a purine-synthesis inhibitor, largely used in association with the calcineurin-inhibitors (CI) to reduce their side effects, in particular renal insufficiency. MMF-monotherapy has been suggested for patients with Orthotopic Liver Transplantation (OLT); however, leucopoenia and anaemia caused by bone-marrow toxicity have to be taken into account.

Methods: Patients with OLT, who were switched from immunosuppressive therapy with CI to MMF, for the development of renal failure, were enrolled in the study. We collected serum values of creatinine, haemoglobin, leucocytes, GGT and AST, related to three different periods of time: the three years before the combination therapy, when the patients were in CI monotherapy (T1); the period of combination therapy (T2) and, finally, the period of MMF-monotherapy (T3).

Results: Eleven patients (median age 65.8 years old (range 34.1–74.8), one female) were included. At the time of the data collection, our patients were in monotherapy with MMF from a median time of 7.8 years (T3), after an average period of 1.2 years of combination therapy (T2). Table 1 summarises the median values (IRQ) of the serum values analysed in the three periods.

Table 1

	T1 Median (IRQ)	T2 Median (IRQ)	T3 Median (IRQ)	p^*
Creatinine mg/dL (vn 0.6–1.2)	1.4 (1.3–1.8)	1.5 (1.1–1.8)	1.4 (1.3–1.6)	0.9
Haemoglobin g/dL (vn 12–15)	13.6 (12.3–14.1)	13.5 (12.3–15.1)	13.9 (12.3–15.4)	0.7
Leucocytes $10^3/\mu L$ (vn 4–10)	5.6 (4.6–6.9)	5.4 (4.3–6.4)	5.8 (4.4–7.2)	0.5
GGT U/l (vn <37)	35 (27–133)	45.5 (26–104)	48 (24–74)	0.9
AST U/l (vn <50)	24 (18–31)	22.5 (17–32)	19 (16–27)	0.06

* Kruskal–Wallis test.

Conclusions: No transplant rejection was noted in patients receiving MMF monotherapy. Renal function remained unchanged through the suspension of the inhibitor of CI, demonstrating that once established renal damage there is no recovery of renal function. In our patients no bone marrow injury was caused by MMF monotherapy.

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2

THE RISK OF HEPATOCELLULAR CARCINOMA IN HBV CIRRHOSIS IS AFFECTED BY POLYMORPHISMS OF THE MERTK GENE

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Introduction: SNPs of the MERTK gene are known to modulate tumor-associated macrophages and may influence angiogenesis and carcinogenesis.

Aims: We assessed the potential association between rs4374383 SNP in the MERTK gene and the risk of development of HCC among patients who had chronic HBV infection with active HBV replication or virological suppression.

Methodology: Enrollment cohort of 327 consecutive patients with chronic HBV infection at first presentation (169 CH; 133 LC; 25 HCC). 248 were given long term treatment with NUCs. SNPs were tested by TaqMan SNP genotyping allelic discrimination (Applied Biosystems, Foster City, CA, USA) on sera.

Results: The overall distribution of SNP rs4374383 alleles was: AA 14.8%, AG 44.4%, GG 40.8%. Among 25 patients with HCC at baseline, 12% were GG and 22 88% GA/AA. Among the remaining 302 patients with CH or LC, 43.3% were GG and 131 56.6% AG/AA (OR: 5.62; 95% CI: 1.65–19.17 p 0.002). In the group on long term NUCs,

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all 248 patients had reached HBV undetectability <20 UI/ml within one year of therapy. Thirty of these patients developed HCC during follow-up. These patients had the GG in 26.7% and the GA or AA 73.3%. Among the 218 who did not develop HCC, 46.3% were GG and 53.7% GA/AA (OR: 2.37; 95% CI: 1.01–5.56 p 0.041). Patients on NUCs with CH had a lower risk of HCC than those with LC (OR: 0.13; 95% CI: 0.04–0.40; p = 0.0001) and cirrhotic patients with GG had a lower risk of HCC than patients with AG/AA (OR: 0.35; 95% CI: 0.13–0.96; p = 0.036).

Conclusions: The AG or AA genotypes of rs4374383 SNP of the MERTK gene confers a significant additional risk of developing HCC to patients with chronic HBV infection, especially in the presence of cirrhosis. Testing patients on NUCs suppressive treatment for this SNP may allow to tailor surveillance for HCC.

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3

CIRCULATING SCCA-IGM COMPLEX IS A USEFUL BIOMARKER TO PREDICT THE OUTCOME OF THERAPY IN HCC PATIENTS

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Background and aims: Every year hepatocellular carcinoma (HCC) develops in about 3–4% of cirrhotic patients. The squamous cell carcinoma antigen (SCCA) was found elevated in liver cancer specimens by immunohistochemistry and detected by ELISA complexed with IgM (SCCA-IgM) in serum of patients with HCC. This study aimed to evaluate the ability of SCCA-IgM serum levels to predict the efficacy of HCC therapy.

Methods: From April 2012 to April 2014, 218 patients (M/F 174/44, median age: 65 years, range 40–82) with a new diagnosis of HCC were enrolled in two referral centers of South Italy, in a prospective study. The diagnosis of HCC was made according to the AASLD 2010 guidelines.

The patients were staged and treated according to BCLC Staging System; in particular, BCLC stage A-B (94 patients) were treated with loco-regional therapy (RF, LTA, PEI, TACE), and BCLC stage C (124 patients) were treated with Sorafenib (800 mg/die). Response to therapy was evaluated with imaging techniques according to the mRECIST criteria.

Serum SCCA-IgM levels were determined by the Hepa-IC kit (Xeptagen, Italy) at basal time. The quantization of SCCA-IgM was expressed in Arbitrary Units (AU/mL).

Results: At the time of diagnosis, SCCA-IgM was reactive in 168/218 patients (77%), mean ± SD: 274.4 ± 263.2 AU/mL. In particular SCCA-IgM was detected in 74/94 (78%) patients with BCLC A-B, and 94/124 (75%) patients with BCLC C, with the mean ± SD value of 258.7 ± 280.5 AU/mL and 295.1 ± 242.5 AU/mL, respectively.

According to mRECIST criteria, 131/168 (78%) patients showed a positive response to therapy; in particular, 66/74 (89%) of BCLC A-B patients, and 65/94 (69%) of BCLC C patients. In non-responder patients (37/168) levels of SCCA-IgM (median: 165.1 AU/mL range: 116.8 ÷ 239.1 AU/mL) were significantly higher than levels measured in patients (131/168) with partial, complete or stationary response to treatment (median: 126.7 AU/mL range: 50.0 ÷ 226.9 AU/mL) ($p < 0.05$).

Conclusions: These results suggest that the determination of SCCA-IgM complex may be helpful in predicting the response to therapy in patients with HCC.

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4

QUALITY OF SLEEP IN PATIENTS WITH LIVER TRANSPLANTATION

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Background: Sleep-related disturbances are increasingly recognized in the setting of chronic liver disease. We aimed to investigate the quality of sleep in patients with Orthotopic Liver Transplantation (OLT).

Methods: Patients with OLT due to cirrhosis, Hepatitis C virus (HCV) or Hepatitis B virus (HBV) correlated, were consecutively enrolled from outpatient clinic at the University of Salerno, from January 2013 and May 2014 and divided in two groups (HCV group and HBV group). Data on sex, age and time from OLT were collected. The participants completed the Pittsburgh Sleep Quality Index (PSQI) to evaluate the quality of sleep. A score higher than 5 was indicative of poor sleep quality. Categorical and continuous variables were expressed as frequency and median with interquartile range (25th, 75th), respectively. Differences in frequencies and differences in non-normally continuous variables were calculated using χ^2 test and Mann–Whitney U test, respectively.

Results: Fifty-six patients were included (34 patients in HCV group and 22 in HBV group). HCV patients were older than HBV patients (median age (IRQ): 64 (60–67) vs 56 (49–58), $p < 0.001$) at the time of the study, but both groups underwent OLT from similar time (median years from OLT (IRQ): 7.5 (3–11) vs 10.5 (6–15), $p = 0.1$). Twelve HCV patients (35.3%) had cirrhosis at the time of the study compared to one patient (4.5%) in the HBV group, $p < 0.001$. Twenty-two HCV patients (64.7%) and 14 HBV patients (63.6%) had a pathological score of PSQI score (>5); the median score of the test was not statistically different between the two groups (median, (IRQ): 6 (4–11) vs 6.5 (4–9), $p = 0.9$).

Conclusions: Patients with OLT showed a poor quality of sleep. The finding was similar for HCV and HBV patients, although HCV patients were older than HBV patients and their long-term survival worse than the HBV ones.

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CHAPTER 4

MERTK rs4374383 polymorphism affect the severity of fibrosis in nonalcoholic fatty liver disease

Research Article

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MERTK rs4374383 polymorphism affects the severity of fibrosis in non-alcoholic fatty liver disease

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Background & Aim: Homozygosity for a common non-coding rs4374383 G>A polymorphism in *MERTK* (myeloid-epithelial-reproductive tyrosine kinase) has been associated with the protection against fibrosis progression in chronic hepatitis C. The main study objective was to assess whether *MERTK* AA genotype influences liver fibrosis, and secondarily *MERTK* expression in patients with non-alcoholic fatty liver disease (NAFLD). We also investigated whether *MERTK* is expressed in human hepatic stellate cells (HSC) and in murine models of fibrogenesis.

Methods: We considered 533 consecutive patients who underwent liver biopsy for suspected non-alcoholic steatohepatitis (NASH) without severe obesity from two Italian cohorts. As controls, we evaluated 158 patients with normal liver enzymes and without metabolic disturbances. *MERTK* rs4374383 genotype was assessed by 5′-nuclease assays. MERTK expression was analysed in mouse models of fibrosis, and the effect of the MERTK ligand GAS6 were investigated in human HSC.

Results: Clinically significant fibrosis (stage F2-F4) was observed in 19% of patients with *MERTK* AA compared to 30% in those with *MERTK* GG/GA (OR 0.43, CI 0.21–0.88, $p = 0.02$; adjusted for centre, and genetic, clinical-metabolic and histological variables). The protective rs4374383 AA genotype was associated with lower MERTK hepatic expression. *MERTK* was overexpressed in the liver of NAFLD patients with F2-F4 fibrosis and in *in vivo* models of fibrogenesis. Furthermore, exposure of cultured human HSC to the MERTK ligand GAS6, increased cell migration and induced procollagen expression. These effects were counteracted by inhibition of MERTK activity, which also resulted in apoptotic death of HSC.

Conclusions: The rs4374383 AA genotype, associated with lower intrahepatic expression of *MERTK*, is protective against F2-F4 fibrosis in patients with NAFLD. The mechanism may involve modulation of HSC activation.

Keywords: NASH; MERTK; Fibrosis.
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Abbreviations: MERTK, myeloid-epithelial-reproductive tyrosine kinase; PNPLA3, patatin-like phospholipase-3; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HOMA, homeostasis model assessment.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease worldwide [1,2], affecting about 20–30% of the general population [3]. The clinical relevance of NAFLD arises from the evidence that NAFLD patients are at risk of progression to cirrhosis and its complications [1,4], presenting also a high rate of cancer and cardiovascular events [5] compared to subjects without fatty liver.

The major risk factors for liver disease severity and its progression are obesity, insulin resistance (IR) and necroinflammation [6–9]. However, several pathogenic factors affecting liver damage in NAFLD are emerging, including immune response, apoptosis, and heritability, which play a key role in the susceptibility towards progressive disease [10]. The patatin-like phospholipase-3 (*PNPLA3*)/adiponutrin rs738409 C>G polymorphism, and the *TM6SF2* rs58542926 G>A polymorphism, are the major common genetic risk factors for NAFLD development and progression [11,12], but their determination is not sufficient to accurately stratify the risk of disease progression at an individual level [12,13].

In this complex picture, a recent genome-wide study in patients with chronic hepatitis C, identified several susceptibility loci for severity and progression of liver fibrosis. The strongest was homozygosity for rs4374383 G>A single nucleotide

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Research Article

polymorphism, a non-coding variant in the myeloid-epithelial-reproductive tyrosine kinase (*MERTK*) locus [14]. MERTK is a receptor of the tumour-associated macrophage (TAM) family, with a key role for the initiation of efferocytosis, a process by which dying cells are removed by phagocytes [15,16]. MERTK was overexpressed in activated mouse hepatic stellate cells (HSC) *in vitro* and in an experimental model of liver fibrosis [17]. Agonists of LXR, a nuclear receptor favouring lipogenesis, increases MERTK expression in monocytes [18], and mice deficient for GAS6, a ligand of MERTK, had an attenuation in hepatic steatosis – via modulating beta-oxidation, inflammation and fibrosis [19]. Therefore MERTK and its variants could act as central players in the control of apoptosis, immune response, HSC activation, and steatosis modulation, i.e. all factors involved in the pathogenesis of NAFLD and of its progression to non-alcoholic steatohepatitis (NASH) and cirrhosis.

The aim of this study was to assess whether the rs4374383 polymorphism influences histological damage (fibrosis as the main objective), and secondarily *MERTK* expression in patients with NAFLD. We also investigated whether MERTK is co-regulated with fibrogenesis, in murine models of fibrogenesis, and its expression in HSC.

Patients and methods

Patients

We analysed data from 533 Italian prospectively recruited patients, who underwent liver biopsy for suspected NASH without severe obesity, and with blood samples available for genetic analyses. The study cohort included 233 patients from the Gastrointestinal & Liver Unit of the Palermo University Hospital, and 300 patients from the Metabolic Liver Diseases outpatient service, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, in Northern Italy. Other causes of liver disease were ruled out, including alcohol intake (>20 g/day) evaluated by a questionnaire, viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha1-antitrypsin deficiency. Patients with advanced cirrhosis, hepatocellular carcinoma, severe obesity (body mass index (BMI) $\geqslant$40 kg/m^2), and current use of steatosis-inducing drugs were excluded.

The control group included 158 Italian subjects; 120 were consecutive healthy blood donors without obesity, diabetes and chronic diseases, and with normal ALT levels (<32/28 U/L in M/F), where NAFLD was rule out by fatty liver index (n = 120) [20]; 38 cases were obese subjects with normal liver biopsy (steatosis <5% of hepatocytes) at routine liver biopsy performed at the time of bariatric surgery (gastric banding). Mean age was 54.2 years, and 46 (29.1%) were females. Mean total and HDL cholesterol, as well as triglycerides were in the normal range, and mean HOMA value was 2.37. All were negative for viral infection (anti-HCV, anti-HIV, and HBsAg negative, viral infection was excluded by determining viremia in blood donors).

The study was carried out in accordance with the principles of the Helsinki Declaration, and with local and national laws. Approval was obtained from the hospital Internal Review Boards and their Ethics Committees, and written informed consent for the study was obtained from all controls and patients.

Clinical and laboratory assessment

Clinical and anthropometric data were collected at the time of liver biopsy. BMI was calculated on the basis of weight in kilograms and height in meters. The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association, using a value of fasting blood glucose $\geqslant$126 mg/dl on at least two occasions [21]. In patients with a previous diagnosis of type 2 diabetes, current therapy with insulin or oral hypoglycemic agents was documented.

A 12-h overnight fasting blood sample was drawn at the time of biopsy to determine serum levels of alanine aminotransferase (ALT), total cholesterol, HDL cholesterol, triglycerides, plasma glucose and insulin concentrations. IR was assessed by homeostasis model assessment (HOMA) using the following equation: Insulin resistance (HOMA-IR) = Fasting insulin (μU/ml) $\times$ Fasting glucose (mmol/L)/22.5. [22].

Genetic analyses

DNA was purified using the QIAmp blood Mini Kit (Qiagen, Mainz, Germany) and DNA samples were quantified using spectrophotometric determination. Genotyping for *PNPLA3* (rs738409 C>G), *TM6SF2* (rs58542926 C>T) and *MERTK* (rs4374383 G>A) variants was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA).

The genotyping call was done with SDS software v.1.3.0 (ABI Prism 7500, Foster City, CA, USA). Genotyping was conducted in a blinded fashion relative to patient characteristics.

Assessment of histology in human NAFLD

Slides were coded and read at each clinical centre by one expert pathologist, who was unaware of the patients' identity and history. A minimum 15 mm length of the biopsy specimen or the presence of at least 10 complete portal tracts was required [23]. Steatosis was assessed as the percentage of hepatocytes containing fat droplets (minimum 5%). The NAFLD clinical research network Kleiner classification [24] was used to compute steatosis, ballooning and lobular inflammation, and to stage fibrosis from 0 to 4. NASH was considered to be present when steatosis, lobular inflammation and ballooning or fibrosis were present.

In 20 cases randomly selected (10 from each centre), we tested the inter-observer agreement between pathologists for fibrosis, steatosis, lobular inflammation and ballooning by using weighted kappa scores.

Evaluation of liver cells expressing MERTK in human NAFLD by immunohistochemical and immunofluorescence analyses

Immunohistochemistry was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometer thick tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was performed using pH9 Tris/EDTA buffer (Novocastra, UK) in a PT Link Dako at 98 °C for 30 min. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H_2O_2 and Fc blocking by a specific protein block (Novocastra) the samples were incubated 1 h with the primary antibodies rabbit monoclonal [Y323] to MERTK anti-human, Abcam code ab52968 (1:1000 pH9) at room temperature. Staining was revealed by polymer detection kit (Novocastra) and revealed by AEC (3-amino-9-ethylcarbazole, Dako, Denmark), substrate-chromogen. The slides were counterstained with Harris hematoxylin (Novocastra).

Immunofluorescence was performed by using the following primary and secondary antibodies: anti-human CD68 (clone PGM1, diluition 1:100, Dako Cytomation); anti-human MERTK [Y323] 1:1000, Abcam). After Fc blocking, primary antibodies binding was revealed by fluorochrome-conjugated secondary antibodies: Alexa 568-conjugated goat anti-rabbit (Invitrogen Molecular Probes, Carlsbad, CA); Alexa 488-conjugated goat anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA). The slides were counterstained with DAPI Nucleic Acid Stain (Invitrogen, Molecular Probes, Carlsbad, CA). All the sections were analysed under a Leica DM3000 optical microscope (Leica Microsystems, Germany) and microphotographs were collected using a Leica DFC320 digital camera (Leica).

Evaluation of MERTK hepatic mRNA levels in human NAFLD

MERTK hepatic expression (mRNA levels) was evaluated by quantitative real-time PCR in available tissue samples immediately frozen at the time of liver biopsy. Liver biopsies were collected in RNAlater (Ambion, Carlsbad, CA, USA), immediately frozen in liquid nitrogen and stored at −80 °C. When an additional tissue sample was available, this was lysed for protein extraction in RIPA buffer, containing 1 mmol Na-orthovanadate, 200 mmol PMSF and 0.02 mg/ml apoprotinin. RNA was isolated from liver biopsies using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer protocol. RNA quality was evaluated by measuring the 260/280 nm absorbance ratio (> 1.8) and by electrophoresis. First-strand complementary DNA (cDNA) was synthesized using SuperScript VILO cDNA synthesis kit (Invitrogen), starting from 0.5 mcg of total RNA. Gene expression was evaluated by quantitative real-time PCR using SYBR Green FAST master mix (Applied Biosystems, Foster City, CA, USA), ran in triplicate on ABI PRISM 7500 fast (Applied Biosystems). Primers sequences used were, for MERTK, forward: 5′-CCTTCAGCATAACCAGTGTGC and reverse: 5′-TGACAGGTGAGGTTGAAGGC, whereas for beta-actin, forward 5′-GGCATCCTCACCCTGAAGTA, reverse: 5′-GGGGTGTTGAAGGTCTCAAA. Gene expression levels were normalized for beta-actin.

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Animal experimental protocol

Two different models of fibrogenesis were employed. Mice were purchased from Charles River Laboratories (Calco, Italy). For carbon tetrachloride (CCl_4)-induced fibrosis, male C57BL/6 mice were injected intraperitoneally with CCl_4 (0.5 μl/g bw) twice a week for 6 weeks to induce liver fibrosis. Control mice received an equivalent volume of olive oil. Mice were sacrificed 2 days after the final CCl_4 injection. Experimental fibrogenic steatohepatitis was induced by administration of a methionine and choline-deficient diet. Male BALB/c mice were fed either a high-fat diet deficient in methionine and choline (MCD diet), or a control diet supplemented with methionine and choline, as previously described [25]. Diets were prepared by Dottori Piccioni Laboratories (Milan, Italy) and stored at 4 °C until used. In both experiments mice, 8 weeks of age at the beginning of this study, weighing between 20 and 25 g were kept under a controlled temperature of 22 ± 2 °C, 50–60% relative humidity and 12 h light/dark cycles. Mice had free access to food and water ad libitum. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985) and experiments were performed after permission of the local IACUC.

Culture of human HSC and measurement of cell migration

HSC were isolated from normal liver tissue unsuitable for transplantation as previously described [26]. Cells were cultured on plastic and used after complete transition to a myofibroblast-like phenotype. Sub-confluent HSCs were serum-starved for 24 h, washed, trypsinized, and re-suspended in serum-free medium at a concentration of 1×10^5 cells/ml. Chemotaxis was measured in modified Boyden chamber equipped with 8 μm pore filters (Millipore Corp, MA, USA) and coated with rat tail collagen (20 μg/ml) (Collaborative Biomedical Products, Bedford, USA), as described in detail elsewhere [26]. At least ten high-power fields per filter were counted in each experiment.

Immunoprecipitation and Western blot analysis

Procedures for preparation of cell lysates, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and Western blotting have been described elsewhere [26]. One hundred μg of cell proteins were used for MERTK immunoprecipitation, performed with 15 μl of anti-MERTK (R&D Systems) or the appropriate control IgG followed by addition of protein A-Sepharose, as described by Di Maira *et al.* [27]. The resulting immunoprecipitates were analysed by Western blotting using anti-MERTK (AbCam). Quantification of the signal was obtained by chemiluminescence detection on an Image Quant Las4000 (GE Healthcare Life Sciences) and subsequent analysis with ImageJ software.

Measurement of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay, as previously described [27].

Gene silencing

All siRNAs used were purchased from Dharmacon (Lafayette, USA). Transfection of human HSC was performed using the Amaxa nucleofection technology (Amaxa, Koln, Germany) as previously described, with 100 nM smart Pool siRNA specific for human *MERTK* (sequence accession no. # NM_006343) or non-targeting siRNA [28].

Statistics

The study had the power to include 12 potential predictors in the multivariate model for F2-F4 fibrosis in a cohort of NAFLD with at least 120 patients with F2-F4 fibrosis, i.e. the expected 25% of the entire cohort. Continuous variables were summarized as mean ± standard deviation, and categorical variables as frequency and percentage. The *t* test and chi-square test were used when appropriate. Univariate and multiple logistic regression models were used to assess the factors independently associated with significant fibrosis (main objective), and with NASH and severe steatosis (secondary objectives). In the first model, the dependent variable was fibrosis, coded as 0 = no or mild fibrosis (F0-F1) or 1 = clinically significant fibrosis (F2-F4); in the second model, the dependent variable was NASH coded as 0 = absent and 1 = present; in the third model the dependent variable was steatosis coded as 0 = mild-moderate (steatosis grade 1–2), and 1 = severe (steatosis grade 3).

As candidate risk factors, we selected age, gender, BMI, the baseline levels of ALT, triglycerides, total and high-density lipoprotein (HDL) cholesterol, blood glucose, insulin, HOMA score, the presence of diabetes, *PNPLA3* rs738409 variant, *TM6SF2* rs58542926 C>T, *MERTK* rs4374383 AA genotype, steatosis, lobular inflammation, ballooning, NASH, fibrosis, and enrolling centre.

In all models, according to literature data, we compared patients homozygous for *MERTK* AA protective allele to all other variants [14], the TM6SF2 variant was coded in a dominant genetic model because of its relatively low allele frequency [12,13], and an additive model was used for *PNPLA3* [11].

To avoid the effect of co-linearity, diabetes, HOMA score, blood glucose and insulin levels, or steatosis, ballooning, lobular inflammation and NASH were not included in the same multivariate model. Regression analyses were performed using SAS [29].

Results

Patients' features

The baseline characteristics of the 233 Sicilian and of the 300 Northern Italian NAFLD patients are shown in Supplementary Table 1. In the group of 20 cases with histological features revised by pathologists from both Northern and Southern Italian centres, the k inter-observer agreements for fibrosis, steatosis grade, lobular inflammation and ballooning were 0.89, 0.76, 0.60 and 0.55, respectively, similar to those reported in the literature [24].

The prevalence of *MERTK* rs4374383 AA, AG and GG genotypes was 14.6%, 47.8% and 37.6% in the entire cohort. Genetic frequencies did not violate with Hardy–Weinberg equilibrium.

The frequency distribution of the *MERTK* genotype was similar in patients with NAFLD respect to age and sex-matched individuals with normal liver enzymes and without metabolic abnormalities ($p = 0.90$; Supplementary Fig. 1). As a positive control, the rs738409 G and rs58542926 T alleles, were over-represented in patients *vs.* controls ($p < 0.0001$).

No associations were found between *MERTK* genotypes and anthropometric, metabolic and biochemical parameters in the entire cohort (Table 1).

Association of MERTK genotype with histological severity of human NAFLD

Clinically significant fibrosis

Clinically significant fibrosis (stage F2-F4) was associated with older age, higher ALT, type 2 diabetes, NASH diagnosis, *PNPLA3* variant and the absence of *MERTK* AA genotype. Specifically, significant fibrosis was observed in 19% of patients with *MERTK* AA compared to 30% of those with *MERTK* GG/GA ($p = 0.04$). By multiple logistic regression analysis, *MERTK* AA genotype remained associated with clinically significant fibrosis after correction for genetic and clinical-metabolic variables (OR 0.40, CI 0.20–0.81, $p = 0.01$), and also for the above indicated factors and histological features of NASH (OR 0.43, CI 0.21–0.88, $p = 0.02$; Table 2, upper panel). Notably, when the term of interaction between *PNPLA3* and *MERTK* variants was included in the model it was not significant ($p = 0.18$).

To account for a possible effect of *MERTK* AA genotype on fibrosis via induction of severe steatosis, *MERTK* AA genotype remained significantly associated with clinically significant fibrosis when NASH was replaced in by severe steatosis in the model (OR 0.46, CI 0.23–0.92, $p = 0.03$).

When considering severe fibrosis (F3-F4) as histological outcome, similar results were observed: *MERTK* AA genotype was

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Research Article

Table 1. Baseline demographic, laboratory, metabolic, and histological features of Italian patients, according to *MERTK* genotype.

	Combined cohort (n = 533)		
	MERTK GG/GA N = 455	*MERTK* AA N = 78	*p* value
Mean age, yr	46.5 ± 11.8	47.3 ± 13.1	0.58
Male gender	61.1	57.7	0.56
Mean BMI, kg/m²	29.9 ± 5.0	29.5 ± 4.6	0.48
ALT, IU/L	58.2 ± 45.0	64.7 ± 72.3	0.29
Type 2 diabetes	17.1	12.8	0.34
Cholesterol, mg/dl	204.1 ± 43.8	212.4 ± 47.6	0.14
HDL cholesterol, mg/dl	49.1 ± 14.7	51.6 ± 16.5	0.17
Triglycerides, mg/dl	145.6 ± 80.7	142.3 ± 76.8	0.74
Blood glucose, mg/dl	97.6 ± 28.1	99.4 ± 25.5	0.72
Insulin, μU/ml	16.4 ± 10.2	16.6 ± 9.4	0.90
HOMA score	4.15 ± 3.36	4.39 ± 3.39	0.62
PNPLA3 GG	15.0	11.5	0.42
PNPLA3 CC/CG/GG	40.4/44.6/15.0	44.9/43.6/11.5	0.64
TM6SF2 CT/TT	13.8	14.2	0.91
Histology			
Lobular inflammation 2-3	22.2	15.4	0.17
Ballooning 1-2	51.6	52.5	0.52
Steatosis grade 3	20.9	7.7	0.006
NASH	44.2	39.7	0.46
Fibrosis stage 2-4	30.3	19.2	0.04

IU, international units; HOMA, homeostasis model assessment; HDL, high density lipoprotein. Data are given as mean ± standard deviation, or as percentage of cases (%).

associated with a lower prevalence of F3-F4 fibrosis after correction for genetic and clinical-metabolic variables (OR 0.37, CI 0.14–0.96, $p = 0.04$) as well as for the above quoted risk factors and NASH (OR 0.38, CI 0.16–0.99, $p = 0.04$).

NASH and severe steatosis

The prevalence of NASH was not affected by *MERTK* genotype (39.7% in *MERTK* AA *vs.* 44.1% in *MERTK* GG/GA; $p = 0.46$), while NASH was linked to higher ALT, type 2 diabetes, *PNPLA3* variant, and enrolling centre. Along this line, by multivariate logistic regression analysis, type 2 diabetes (OR 2.16, 95% C.I. 1.11–4.19 $p = 0.02$) and Southern Italian enrolling centre (OR 31.9, 95% C.I. 19.0–53.6; $p < 0.001$) were independently linked to NASH, but not *MERTK* AA genotype (OR 0.63, 95% C.I. 0.31–1.30; $p = 0.21$).

Severe steatosis was observed in 8% of patients with *MERTK* AA compared with 21% with *MERTK* GG/GA genotype ($p = 0.006$). The other variables associated with severe (grade 3) steatosis were higher BMI, higher ALT levels, both *PNPLA3* and *TM6SF2* variants, and enrolling centre. By multivariate logistic regression analysis *MERTK* AA genotype (OR 0.24, 95% C.I. 0.09–0.63; $p = 0.004$) remained significantly associated with severe steatosis together with ALT levels (OR 1.00, 95% C.I. 1.00–1.01; $p = 0.007$), BMI (OR 1.11, 95% C.I. 1.05–1.17; $p < 0.001$), *PNPLA3* variant (OR 2.09, 95% C.I. 1.49–2.93; $p < 0.001$), *TM6SF2* variant (OR 2.64, 95% C.I. 1.45–4.81; $p = 0.002$), and Southern Italian enrolling centre (OR 3.03, 95% C.I. 1.79–5.12; $p < 0.001$).

MERTK is expressed in hepatic stellate or monocytoid cells from human NAFLD samples

MERTK was found to be expressed in cells with stellate or monocytoid morphology scattered throughout the hepatic parenchyma and loosely aggregated within inflammatory foci (Fig. 1, upper panels) while being not expressed in hepatocytes. Since the morphology of MERTK-expressing cells was suggestive of stellate cells/macrophages double labelling immunofluorescence analysis was performed for the CD68 macrophage marker and MERTK. Consistent with the morphology observed on immunohistochemically-stained sections, MERTK-expressing cells also expressed CD68 (Fig. 1, lower panels), which confirmed their macrophagic lineage.

Hepatic expression of MERTK in patients with NAFLD

In 94 morbidly obese consecutive patients from Northern Italy (male gender 30%, mean age 44 ± 11 years, mean BMI 41 ± 8 kg/m^2), and at a very low prevalence of F2-F4 fibrosis, 13% [30], the protective rs4374383 AA genotype was associated with lower *MERTK* expression levels ($p = 0.049$; Fig. 2A), after correction for age, gender, and lobular inflammation (factors linked with *MERTK* expression with $p < 0.1$ at univariate analysis together with *MERTK* genotype). Similarly, in 80 NAFLD patients from the Southern Italian cohort (male gender 30%, mean age 48 ± 11 years, mean BMI 29 ± 4 kg/m^2, F2-F4 fibrosis 42.5%) we confirmed lower *MERTK* expression levels in those carrying the *MERTK* AA genotype compared with all the others ($p = 0.02$; Fig. 2B).

To further establish a possible link between *MERTK* expression and fibrogenesis in NAFLD, hepatic *MERTK* expression was assessed in a function of fibrosis severity in 27 patients from the FLIP study (male gender 75%, mean age 42 ± 11, mean BMI 27 ± 4), and in the above quoted Southern Italian cohort of 80 patients with NAFLD. Of note, patients with F2-F4 fibrosis had significantly higher *MERTK* mRNA levels than those with F0-F1 in both FLIP and Southern Italian cohorts (Fig. 2C, D). No association was found between hepatic *MERTK* expression and other histological features (data not shown).

MERTK expression is upregulated in murine models of fibrosis

We also investigated the expression of *MERK* in two well-established models of fibrogenesis in the mouse (Fig. 3). After a 6-week administration of CCl_4, intrahepatic expression of *MERTK* was more than 6-fold higher than in control mice. Similarly, 8-week administration of a MCD diet resulted in a 2.5-fold increase in *MERTK* mRNA levels ($p < 0.05$). This latter model is more representative of fibrogenesis associated with steatohepatitis, and the lower degree of *MERTK* expression reflects the less abundant fibrosis observed in this model. We also tested hepatic *MERTK* expression in a murine high-fat diet (HFD) model showing at 16 weeks no different expression of *MERTK* compared to controls (data not shown). Overall, these data provide additional support to the profibrogenic role of *MERTK* in models driven by inflammatory and metabolic/inflammatory pathways.

Fibrogenic effects of a MERTK ligand in cultured stellate cells

HSC represent the final effectors and coordinators of the fibrogenic response, and we explored whether *MERTK* could affect the biology of this cell type. Based on the evidence that *MERTK* mRNA expression was significantly induced in mouse HSC activated by culture in plastic, and also after induction of chronic liver damage in response to CCl_4 administration or bile duct ligation [17], we analysed whether activation of *MERTK* was

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Table 2. Association of the *MERTK* rs4374383 genotype and liver damage as evaluated by unadjusted and adjusted models in 533 patients with biopsy-proven non-alcoholic fatty liver disease.

Variable	Significant fibrosis		
	Unadjusted model[&]	Adjusted model 2*	Adjusted model 3[§]
	OR (95% CI) *p* value		
Mean age, yr	1.03 (1.02-1.05) <0.001	1.05 (1.02-1.07) <0.001	1.04 (1.02-1.06) <0.001
ALT, IU/L	1.01 (1.00-1.01) <0.001	1.01 (1.00-1.01) 0.001	1.00 (1.00-1.01) 0.003
Type 2 diabetes	3.02 (1.92-4.93) <0.001	2.03 (1.16-3.54) 0.01	1.91 (1.06-3.45) 0.03
NASH	10.7 (6.78-17.0) <0.001	-	7.19 (3.81-13.5) <0.001
MERTK GG/GA *vs.* AA	0.54 (0.30-0.99) 0.04	0.40 (0.20-0.81) 0.01	0.43 (0.21-0.88) 0.02
PNPLA3 CC *vs.* CG *vs.* GG	1.29 (0.99-2.69) 0.05	1.05 (0.77-1.43) 0.75	1.01 (0.73-1.40) 0.90
TM6SF2 CC *vs.* CT/TT	1.23 (0.72-2.08) 0.44	1.26 (0.69-2.32) 0.44	1.28 (0.67-2.43) 0.44
Southern Italian cohort	5.58 (3.69-8.46) <0.001	5.07 (3.19-8.08) <0.001	1.41 (0.75-2.66) 0.27

[&]Genetic, clinical-metabolic, and histological variables significant at univariate analyses were included in the multivariate logistic regression model. TM6SF2 variant was forced in the model.

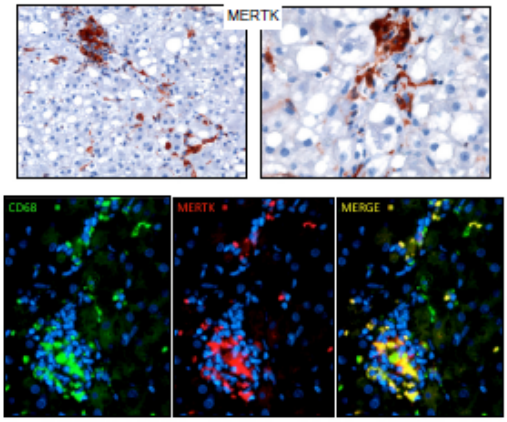

Fig. 1. MERTK *in situ* hepatic expression in human NAFLD. MERTK *in situ* expression was investigated by immunohistochemistry on NAFLD bioptic samples using a specific monoclonal primary antibody. MERTK was found to be expressed in cells with stellate or monocytoid morphology scattered throughout the hepatic parenchyma and loosely aggregated within inflammatory foci (upper panels) while being not expressed in hepatocytes. Since the morphology of MERTK-expressing cells was suggestive of stellate cells/macrophages double labelling immunofluorescence analysis was performed for the CD68 macrophage marker and MERTK. Consistent with the morphology observed on immunohistochemically-stained sections, MERTK-expressing cells also expressed CD68 (lower panels), which confirmed their macrophagic lineage.

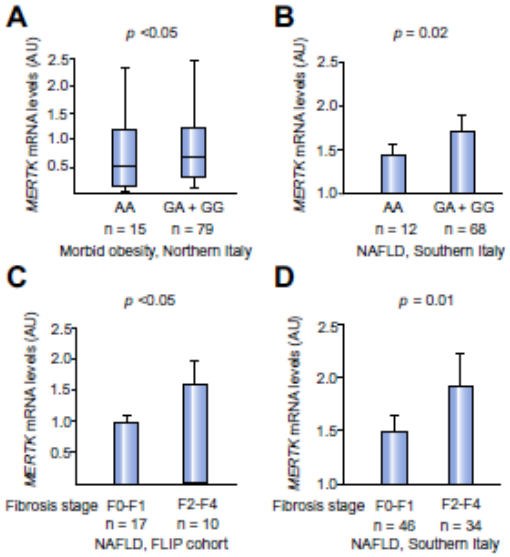

Fig. 2. Hepatic *MERTK* mRNA expression. *MERTK* mRNA hepatic levels according to *MERTK* rs4374383 genotype in 94 bariatric severe obese patients from Northern Italy (A), and in 80 NAFLD patients from Southern Italian cohort (B); *MERTK* mRNA hepatic levels according to presence/absence of F0-F2 fibrosis in 27 NAFLD patients from FLIP cohort (C), and in 80 NAFLD patients from Southern Italian cohort (D).

expressed in cultured human HSC and if its activation modifies the fibrogenic phenotype of these cells. Immunoprecipitation of HSC lysates with anti-MERTK antibodies and blotting for MERTK revealed a clear band of the expected molecular weight, which was not present when lysates were incubated with non-immune, control antibodies (Fig. 4A). Exposure of human HSC activated by culture on plastic to GAS6, a MERTK ligand, induced a time-dependent increase in the activation of ERK1/2, a member of the mitogen-activated protein kinase family activated by different transmembrane receptors including tyrosine kinases and involved in HSC activation and migration (Fig. 4B). In line with these findings, exposure to GAS6 resulted in an increase in cell migration (Fig. 4C), to an extent similar to that induced by foetal bovine serum, used as a positive control. Collectively, these data indicate that culture-activated HSC express functional MERTK.

We next evaluated the specificity of the effects of GAS6 using UNC569, a specific small molecule inhibitor of MERTK [31]. Induction of cell migration in response to GAS6 was reverted in HSC co-treated with UNC569 (Fig. 5A), providing evidence that the effects of GAS6 are mediated by MERTK. Exposure of HSC to UNC569 also resulted in a significant decrease in cell viability, indicating that MERTK is involved in the maintenance of

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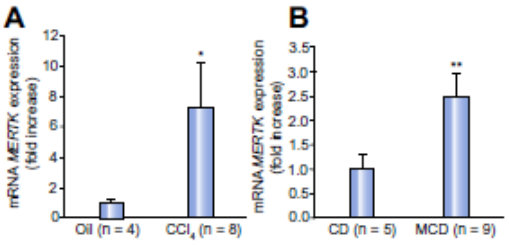

Fig. 3. Mice hepatic *MERTK* mRNA expression. (A) Male mice were injected intraperitoneally with a dose of CCl_4 (0.5 μl/g body weight) or olive oil twice a week for 6 weeks, *p <0.05 *vs.* oil. (B) Male mice were administered with a control diet (CD) or a diet without methionine and choline (MCD) for 8 weeks, **p <0.05 *vs.* CD.

fibrogenic cell survival (Fig. 5B). To rule out that the effects of UNC569 were due to non-specific inhibition of other molecules, we silenced *MERTK* using specific siRNAs. Also with this approach, viability of HSC was significantly reduced, essentially replicating the effects of UNC569 (Fig. 5C). To establish whether the reduction of cell viability was due to programmed cell death, HSC were exposed to increasing concentrations of UNC569 (Fig. 5D). Similar to the effects of doxorubicin in HuH7 cells, used as a positive control, UNC569 resulted in PARP cleavage and in the generation of active caspase-3, compatible with induction of HSC apoptosis.

To obtain further evidence for a profibrogenic role of MERTK, GAS6-stimulated cells were assayed for procollagen I gene expression in the presence or absence of UNC569 (Fig. 5E). GAS6 slightly, but significantly increased the expression of pro-collagen I. These effects were inhibited by co-exposure of HSC to UNC569, indicating that the effects of GAS6 are mediated by MERTK (Fig. 5E). A reduction in procollagen I gene expression was also obtained after *MERTK* silencing (data not shown). Taken together, these data indicate a profibrogenic role for MERTK activation.

Discussion

The main finding of this study is that the *MERTK* rs4374383 AA genotype is associated with a lower prevalence of clinically significant fibrosis in patients with NAFLD, and the mechanism may be mediated by decreased expression of *MERTK*. We also showed that MERTK is expressed in human HSC at the protein level, where mediates profibrogenic actions, and is overexpressed in patients with NASH and severe fibrosis, and in murine models of fibrogenesis.

MERTK is a TAM receptor known to be highly expressed in M2-polarized macrophages, and has a key role in efferocytosis [15,16]. In genome-wide association studies, the rs4374383 gene variant of *MERTK* has been recently associated with liver fibrosis progression in patients with chronic hepatitis C [14], this association being confirmed in another independent cohort of chronic hepatitis C patients [32]. To the best of our knowledge, this is the first study to demonstrate the potential association between MERTK polymorphisms and the fibrotic phenotype in NAFLD patients, and to show a potential direct profibrogenic action.

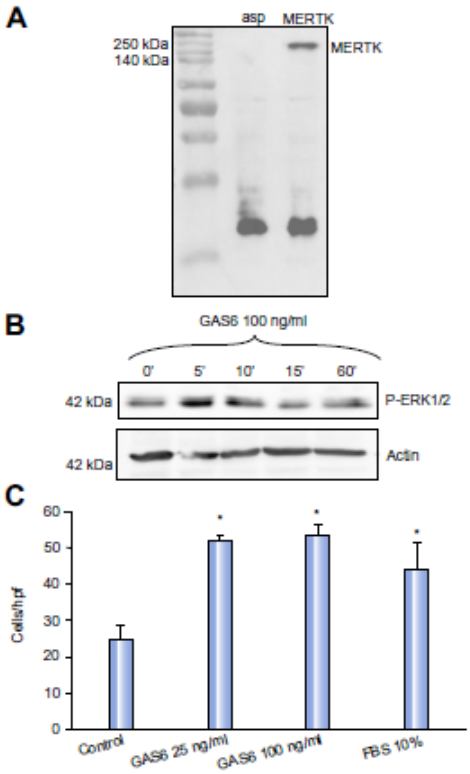

Fig. 4. Role of MERTK protein on primary HSCs motility and ERK activation. (A) MERTK expression was detected by immunoprecipitation with anti-MERTK or aspecific antibody, as indicated, from HSC lysate. Precipitated proteins were detected by Western blot with anti-MERTK. (B) Cells were serum-starved for 24 h and then treated with 100 ng/ml of GAS6 at different times as indicated in the figure. Total cell lysates from primary HSCs were analysed by Western blotting using the indicated antibodies. (C) Migration in the presence or absence of the indicated concentrations of GAS6 for 6 h were measured using modified Boyden chambers. As positive control cells were stimulated with 10% foetal bovine serum for the same time. *p <0.05 *vs.* control.

The most relevant result from our analysis is the protective effect of the *MERTK* AA genotype on significant fibrosis. Notably, this association was maintained after correction for well known clinical-metabolic risk factors, and for both *PNPLA3* and *TM6SF2* gene variants, the strongest genetic determinant of NAFLD [11,12]. Another relevant finding of our study is the association of *MERTK* AA genotype and lower prevalence of severe steatosis in NAFLD patients. Therefore, we suggest that the AA genotype may represent a moderator of the effect of other risk factors on steatosis severity in NAFLD. Published genome-wide association studies assessing gene variants at risk for NAFLD did not identify *MERTK* rs4374383 G>A as one at risk. This data are not in contrast with the results of the present study, because we confirmed *MERTK* gene variant as not associated with NAFLD, while we firstly report in this clinical setting its role as a modulator of liver damage.

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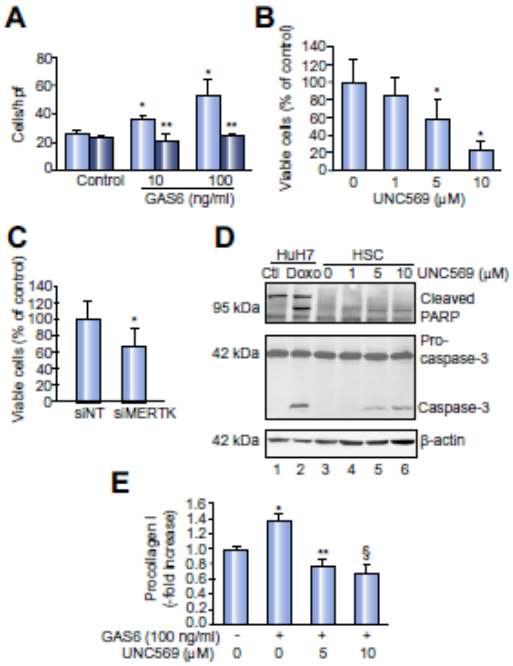

Fig. 5. Effects of a MERTK inhibitor or of MERTK knockdown on the biology of HSC. (A) Migration of HSC in response to the indicated concentrations of GAS6 and in the presence (dark blue columns) or absence (light blue columns) of 5 μM UNC569 was measured using Boyden chambers. *p <0.05 *vs.* unstimulated control; **p <0.05 *vs.* the same GAS6 concentration without inhibitor, hpf, high power field. (B) Serum-deprived HSC were incubated in the presence of the indicated concentrations of GAS6 for 48 h. Cell viability was measured as described in Materials and methods. *p <0.05 *vs.* control (no UNC569). (C) HSC were transfected with MERTK-specific siRNAs or with non-targeting (siNT) siRNAs, as described in Materials and methods. *p <0.05 *vs.* non-targeting siRNAs. (D) Lanes 1–2: Cultured HuH7 were incubated with 10 μg/ml doxorubicin or its vehicle for 48 h. Lanes 3–6: serum-deprived HSC were incubated in the presence of the indicated concentrations of UNC569. Total cell lysates were analysed by Western blotting using the indicated antibodies. (E) Serum-deprived HSC were incubated with the indicated concentrations of GAS6 and in the presence or absence of UNC569 for 48 h. At the end of the experiment gene expression of type I procollagen was measured as indicated in Materials and methods. *p<0.05 *vs.* unstimulated control; **p <0.05 *vs.* GAS6 without inhibitor; § p = 0.07 *vs.* GAS6 without inhibitor.

Although this study was not designed to fully clarify the pathogenic link between *MERTK* AA genotype and the severity of liver fibrosis, several hypotheses may be put forward to mechanistically explain this association. In NAFLD patients, where *MERTK* is mostly expressed in macrophage and HCS, we observed a significantly lower hepatic expression of *MERTK* in subjects carrying the protective AA genotype, which may therefore have a functional counterpart on the abundance of this receptor. Along this line, we found higher hepatic expression of *MERTK* in NAFLD patients with significant fibrosis compared to those with none or mild fibrosis (F0-F1). Remarkably, increased expression of *MERTK* was also present in two independent murine models of fibrogenesis, one of which – the MCD diet – is associated with a histological picture similar to the one of human NASH, while *MERTK* was not overexpressed in the HFD model (data not shown), where the role of inflammatory cells in liver damage is minimal.

A very intriguing finding is that MERTK may modulate the fibrogenic process via direct actions in HSC. Gene expression profiles of HSC undergoing transactivation showed that *MERTK* is induced more than 4-fold following activation on plastic, and *MERTK* hepatic expression is also induced by chronic liver injury *in vivo* [17]. We demonstrated herein that cultured human HSC express a robust signal for MERTK at the protein level. Moreover, we provide additional, functional data on a possible direct action of this tyrosine kinase, exposing HSC to the MERTK ligand GAS6. GAS6 resulted in activation of the ERK1/2 pathway, coupled with induction of directional migration of HSC. Both these actions are relevant for the fibrogenic process, as indicated by the accumulation of activated HSC in discrete regions of the hepatic acinus during fibrogenesis and by the observation that the activation of ERK1/2 positively modulates fibrosis [33]. Moreover, GAS6 induced a significant increase in the expression of type I procollagen. It should be considered that GAS6 is also a ligand of the tyrosine kinase Axl, the expression of which has been previously reported in HSC and in liver fibrosis [34]. To rule out that the effects of GAS6 were not mediated by MERTK, we tested the effects of the specific inhibitor UNC569 [31]. This compound prevented GAS6-mediated effects on cell migration and procollagen expression, indicating that MERTK contributes to mediate the actions of this soluble mediator on HSC. Moreover, UNC569 resulted in a marked reduction in HSC viability via the induction of apoptosis, indicating that MERTK is implicated in the maintenance of HSC survival. Of note, these effects of the MERTK inhibitor were reproduced by genetic *MERTK* silencing. Further studies in *in vivo* models are needed to better define the role of this pathway in the pathogenesis of fibrosis in general and during NASH. When considering the association between *MERTK* variant and the severity of steatosis, data from the literature suggest that *MERTK* may modulate the mechanisms regulating lipogenesis. First, agonists of LXR, a nuclear receptor involved in the regulation of lipid metabolism, increase MERTK expression in monocyte [18]; second, in mice fed a choline-deficient ethionine-supplemented diet (CDE) GAS6 deficiency attenuated hepatic steatosis, limiting CDE-induced downregulation of genes involved in β-oxidation [19].

If further confirmed in independent cohorts, the association between the *MERTK* rs4374383 AA genotype and severity of histological features in NAFLD that may further refine our ability to identify patients needing histological evaluation, or, after prospective studies, those at risk of liver disease progression and mortality. Furthermore, the kinase domain of MERTK may represent a therapeutic target to inhibit disease progression.

Some limitations of this study must be acknowledged, such as its cross-sectional nature, which makes it impossible to dissect the temporal relations between the genetic background and progression of liver disease over time. A further methodological question is to what extent these data may be extrapolated to different populations. Our study included a cohort of Italian patients enrolled at two tertiary care centres, which may be different, in terms of both metabolic features and severity of liver disease, from the majority of prevalent cases of NAFLD in the general population and/or in different geographical areas. Along this line another potential limitation is related to the relative heterogeneity of the study cohorts, relative to the different prevalence of metabolic comorbidities and of severity of liver damage between

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CHAPTER 5
SUMMARY AND GENEREL DISCUSSION

5. SUMMARY AND GENERAL DISCUSSION

In **chapter 2**, we evaluated a prospective cohort of 349 patients with compensated HCV cirrhosis, treated with Peg-Interferon alfa-2b and ribavirin.

Among 349 patients, the AA rs4374383 genotype was reported in 16.9%, the GA genotype in 46.4% and the GG genotype in 36.9%. Of whole cohort, only 87 patients achieved a Sustained Virological Response (SVR).During follow-up, 6.8% of the patients with SVR and 25.2% of those without SVR developed HCC.

The variables independently associated to development of HCC were the age (HR:1.04), the absence of SVR (HR:3.31) and the genotype AA of rs4374383 SNP (HR:2.67) (Tab. 1).

	No HCC 277 pts (79.4%)	HCC 72 pts (20.6%)	p value	Adjusted HR (95%CI)	p value
Age (years, mean, SD)	57.7 ± 9.0	60.3 ± 6.8	0.008	1.04 (1.01-1.07)	**0.045**
Gender (% males)	168 (60.6%)	49 (68.1%)	0.248		
ALT (IU/L, mean, SD)	148.2 ± 86.8	140.0 ± 74.8	0.557		
GGT (IU/L, mean, SD)	79.1 ± 42.9	95.6 ± 62.8	0.025	1.14 (1.20 - 1.37)	**0.008**
Platelets (x 10^9/L)	115.5 ± 45.8	106.5 ± 44.2	0.135		
Prothrombin time (mean,SD)	88.4± 13.2	85.8 ± 16.7	0.230		
Bilirubin (mg/dL, mean,SD)	1.0 ± 0.5	1.0 ± 0.6	0.718		
Albumin (g/dL, mean, SD)	4.0 ± 0.5	3.9 ± 0.5	0.061	0.89 (0.51 – 1.57)	0.689
MERTK polymorphism (*)					
SNP GG	111 (40.0%)	17 (23.6%)			
AG	127 (45.8%)	35 (48.6%)		1.74 (0.95 - 3.18)	0.072
AA	39 (14.2%)	20 (27.8%)	0.005	2.67(1.36 - 5.23)	**0.004**
Esophageal varices	136 (49.1%)	41 (56.9%)	0.282		
No SVR	196 (70.8%)	66 (91.7%)	<0.001	3.31 (1.43 – 7.68)	**0.005**

Table 1. Risk factors for HCC by Cox multivariate model.

By evaluating the incidence of HCC, according to SVR and genotypes of rs4374383 SNP, we have been found that the risk of developing HCC was higher in the patients with HCV cirrhosis, non responders to antiviral therapy and carrying genotype AA compared with whose without SVR and with genotype GG or GA (Fig. 6).

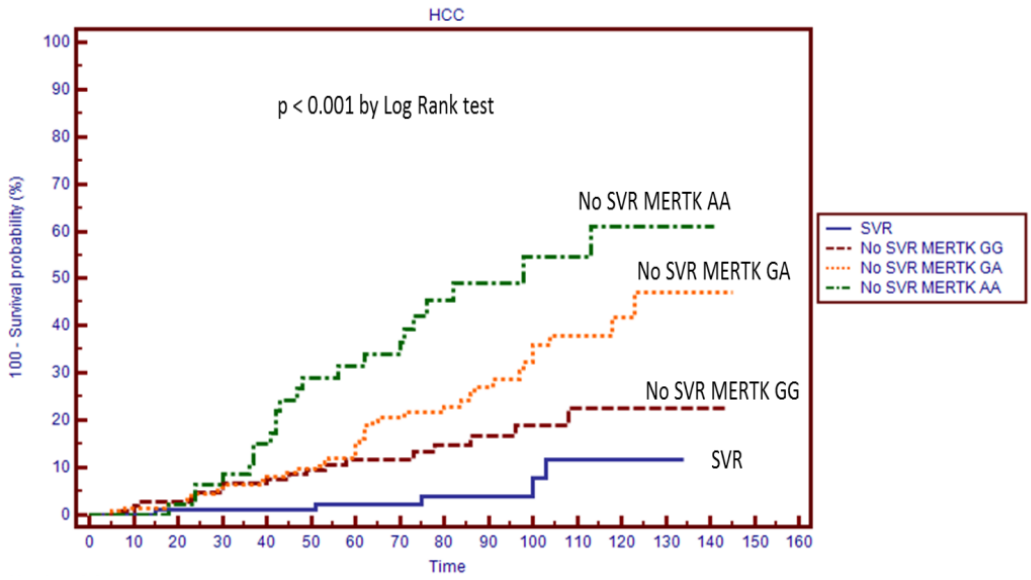


Figure 6. Incidence of HCC in HCV cirrhosis according to SVR and genotypes of the SNP rs4374383 in the MERTk gene.

This study showed that the AA genotype of MERTK if on one the hand confers a low risk of developing fibrosis, on the other is associated with a high risk of HCC development.

MerTK receptor is a regulator of tumor-associated macrophages (TAMs)involved not only in efferocytosis but also in the progression of cancer (Mantovani a., et al., 2011).To comprise if the association between MERTK polymorphism and HCC development was virus-dependent or was related to the inflammatory response and to mechanisms of angiogenesis or tumorigenesis, we studied patients with chronic liver disease from etiology other than HCV infection.

For this purpose, in **chapter 3**, we evaluated MERTK polymorphism in a cohort of 554 patients with chronic HBV infection, at first presentation. The DNA of only 327 patients was available for genotyping.

Among 327 HBV patients, 169 presented a chronic hepatitis (CHB), 133 were cirrhotic whereas the remaining 25 showed HCC.

The distribution of the rs4374383 genotypesamong the 302 patients without HCC was respectively: 14.8% AA genotype, 44.4% AG genotype and 40.8% GG genotype.In the 25 patients with HCC at baseline, 3 (12%) were GG and 22 (88%) were GA or AA.

Between the 327 patients, only 248 were given long term treatment with NUCs; among these, during follow-up, 30 (26.7% and 73.3% respectively with GG and with GA/AA genotype) developed HCC whereas the remaining 218 (46.3% and 53.7% respectively with GG and GA/AA genotype) not developed the cancer.

By evaluating the incidence of HCC, in according to the disease stage and the genotypes of rs4374383 SNP, the probability of HCC development is higher in the patients with LC and genotypeAA/GA respect to patients with LC and genotype GG.

In addition, the incidence of HCC development in CHB patients without cirrhosis and with GA/AA or GG genotype respectively was lower and similar respect to those with cirrhosis (*Fig. 7*).

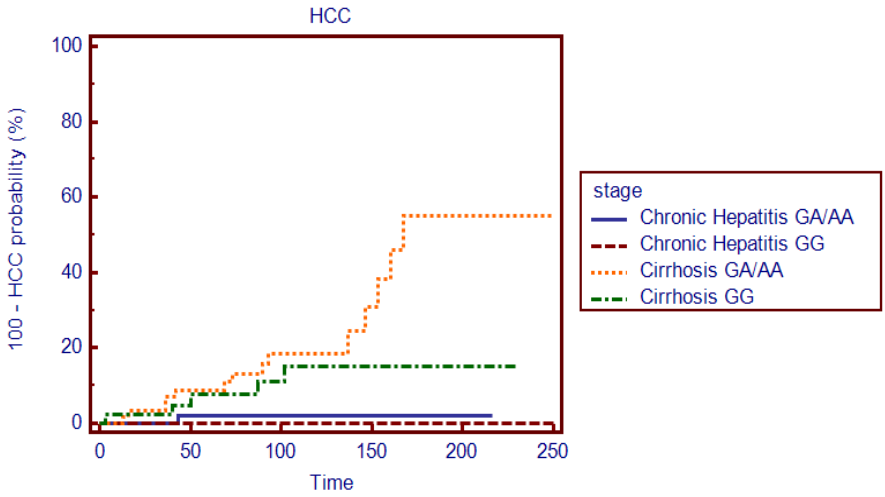


Figure 7: Incidence of HCC in patients with chronic HBV infection, according to disease stage and genotypes of the SNP rs4374383 in the gene MERTK.

Thus, the A allele, in homoor in eterozygosity, confers a significant additional risk for HCC developing in patients with chronic HBV infection, especially in presence of cirrhosis.

These results confirm that the association between MERTK polymorphism and HCC is virus-independent and correlates with the inflammatory response and probably with the mechanisms of angiogenesis and tumorigenesis.

In addition, we have been found that, if on one hand, AA/GA genotype of rs4374383 SNP, is protective respect tofibrosis progression(Patin et al., 2012), on the other the A allele alone confers a significant additional risk for HCC.

Since the inflammatory status is a feature of chronic liver disease and of its complications, in **chapter 4,**we investigated about the possible influence of MERTK gene polymorphism on the progression of fibrosis and its complicationsin two combined cohorts of 533 patients with NAFLD. In addition, in one subgroup of NAFLD we investigated the MERTK liver expression and in murine models of fibrogenesis the MERTK expression in human HSC.

Among 533 patients,who were been underwent liver biopsy for suspected NASH without severe obesity, 37.6% presented GG genotype, 47.8% GA genotype and 14.6% AA genotype.

A significant fibrosis was observed only in 19.2 % of patients with AA genotype respect to 30.3% of those which presented GG or GA genotype. The AA genotype was associated also with clinically significant fibrosis and histological features of NASH (*Table 2*).

About the severe fibrosis (F3-F4) as histological outcome, the AA genotype was associated with a lower prevalence of F3-F4 fibrosis and NASH.

The prevalence of NASH was not affected by MERTK genotype: indeed, NASH was observed in 39.7% of patients with AA genotype and in 44.2% of those with GG or GA genotype (*Table 2*).

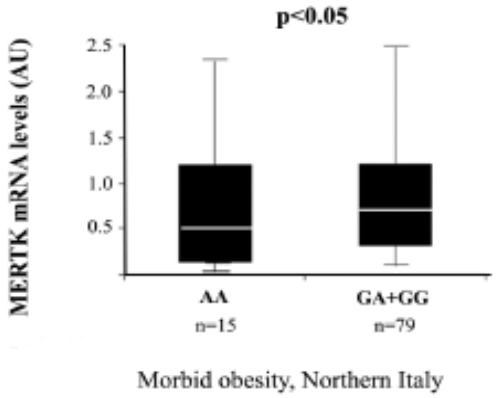
Severe steatosis was observed in 7.7% of patients with AA genotype against the 21% of those with GG or GA genotype (*Table 2*). This result suggests that, in NAFLD, the AA genotype can represent a moderator of the effect of the other risk factors (e.g. immune response, apoptosis and hereditability such as PNPLA3 rs738409 C>G gene) that influence the severity of steatosis (Petta S, et al., 2015).

	Combined cohort (n = 533)		
	MERTK GG/ GA N = 455	*MERTK* AA N = 78	*p* value
Mean age, yr	46.5 ± 11.8	47.3 ± 13.1	0.58
Male gender	61.1	57.7	0.56
Mean BMI, kg/m²	29.9 ± 5.0	29.5 ± 4.6	0.48
ALT, IU/L	58.2 ± 45.0	64.7 ± 72.3	0.29
Type 2 diabetes	17.1	12.8	0.34
Cholesterol, mg/dl	204.1 ± 43.8	212.4 ± 47.6	0.14
HDL cholesterol, mg/dl	49.1 ± 14.7	51.6 ± 16.5	0.17
Triglycerides, mg/dl	145.6 ± 80.7	142.3 ± 76.8	0.74
Blood glucose, mg/dl	97.6 ± 28.1	99.4 ± 25.5	0.72
Insulin, μU/ml	16.4 ± 10.2	16.6 ± 9.4	0.90
HOMA score	4.15 ± 3.36	4.39 ± 3.39	0.62
PNPLA3 GG	15.0	11.5	0.42
PNPLA3 CC/CG/GG	40.4/44.6/15.0	44.9/43.6/11.5	0.64
TM6SF2 CT/TT	13.8	14.2	0.91
Histology			
Lobular inflammation 2-3	22.2	15.4	0.17
Ballooning 1-2	51.6	52.5	0.52
Steatosis grade 3	20.9	7.7	0.006
NASH	44.2	39.7	0.46
Fibrosis stage 2-4	30.3	19.2	0.04

IU, international units; HOMA, homeostasis model assessment; HDL, high density lipoprotein. Data are given as mean ± standard deviation, or as percentage of cases (%).

Table 2. Baseline demographic, laboratory, metabolic and histological features of Italian patients, according to MERTK genotype.

The hepatic expression of MERTK, was evaluated in 94 morbid obese consecutive patients from Northern Italy.Among these, 13.8%, characterized by very low prevalence of F2-F4 fibrosis, showed the protective AA genotype which was associated with lower MERTK expression levels (Petta S, et al., 2015) (*Fig. 8*).



Morbid obesity, Northern Italy

Figure 8.Prevalence of F2-F4 fibrosis and MERTK expression levels in relationship with the genotype MERTK in 94 morbid obese patients from Northern Italy.

Similarly, in 80 NAFLD patients from the Southern Italian cohort, 15% of subject carrying AA genotype, showedlower MERTK expression associated to very low prevalence of F2-F4 fibrosis, compared with all the others (Petta S, et al., 2015) (*Fig.9*).

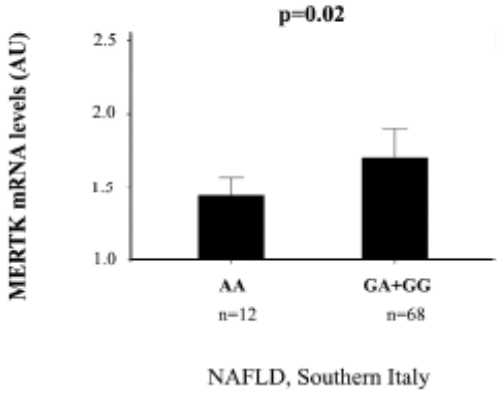


Figure 9.Prevalence of F2-F4 fibrosis and MERTK expression levels in relationship with the genotype MERTK in 80 NAFLD patients from Southern Italian cohort.

Finally, to establish a possible link between MERTK expression and fibrogenesis in NAFLD, the hepatic MERTK expression was assessed in function of fibrosis severity in 27 patients from the FLIP cohort and in the same Southern Italian cohort of 80 patients NAFLD.

In both FLIP and Southern Italian cohorts, the patients with F3-F4 fibrosis showed significantly higher MERTK mRNA levelsthan those with F0-F1 (*Fig. 10 and 11*).

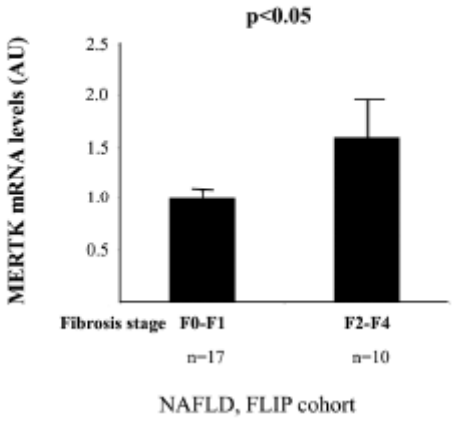


Figure 10. Relationship between MERTK expression and fibrogenesis in 27 patients from FLIP cohort.

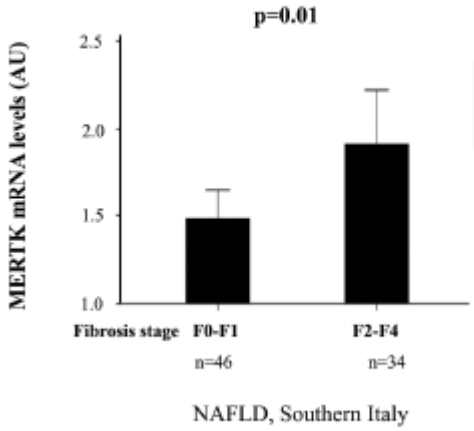


Figure 11. Relationship between MERTK expression and fibrogenesis in 80 NAFLD patients from Southern Italian cohort.

We also investigated the expression of MERTK in two murine models of fibrogenesis.

In the first model, after a 6-week administration of CCl4, the hepatic expression of MERTK was more 6-fold higher than in control mice (*Fig.12*).

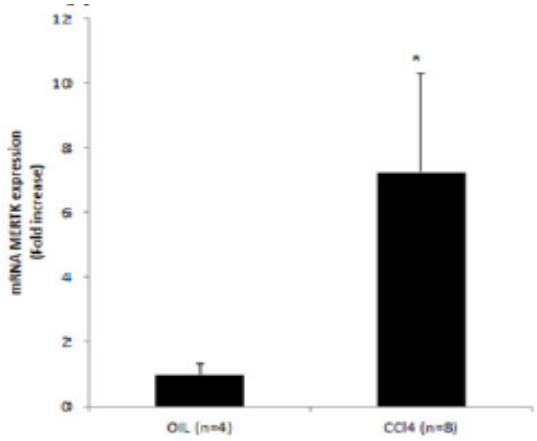


Figure 12.MERTK mRNA levels in a well-established model of fibrogenesis in mouse after administration of CCl4.

Similarly, in the second model, after a 8-week administration of a MCD diet, the hepatic expression of MERTK increased 2.5-fold (*Fig. 13*).

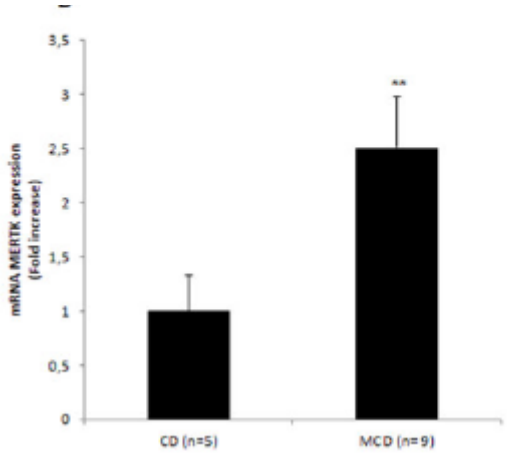


Figure 13.MERTK mRNA levels in a well-established model of fibrogenesis in mouse after administration of a MCD diet.

The high degree of MERTK expression in these murine models provide additional support to the profibrogenic role of MERTK in a context where are involved metabolic and inflammatory pathways (Petta S, et al., 2015).

In addition to these results, we analyzed if the MERTK mRNA expression was followed by its production, in terms of protein, and if MerTK was able to modify the fibrogenic phenotype of these cells. The immune-precipitation of the lysates of the HSC with anti-MERTK antibodies and blotting for MERTK, revealed a clear band of the expected molecular weight, which was not present when lysates were incubated with control antibodies (*Fig. 14*).

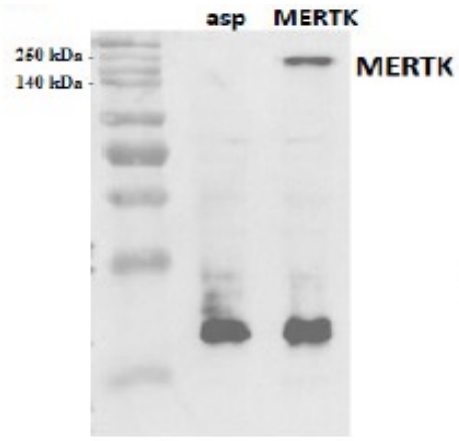


Figure 14. Activation of MERTK in cultured human HSC.

To comprise the effect of this activation, we exposed human HSCs, activated by culture on plastic, to Gas6. This exposure induced a rapid increase of the activation of ERK1/2 (Fig. 15).

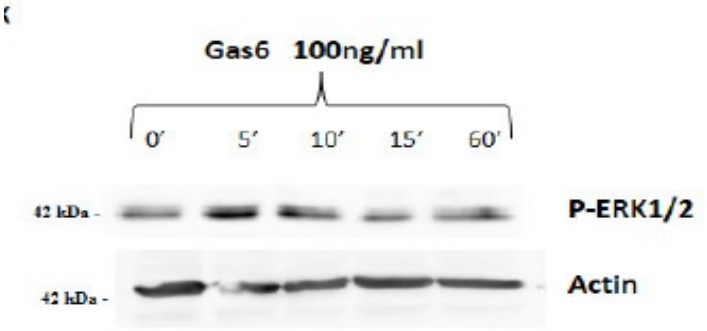


Figure 14. Exposure of human HSC at Gas6 and induction of the increase of the activation, of ERK1/2.

The Exposure to Gas6 resulted also in an increase in cell migration indicating that, culture-activated HSC, express functional MERTK(*Fig. 15*).

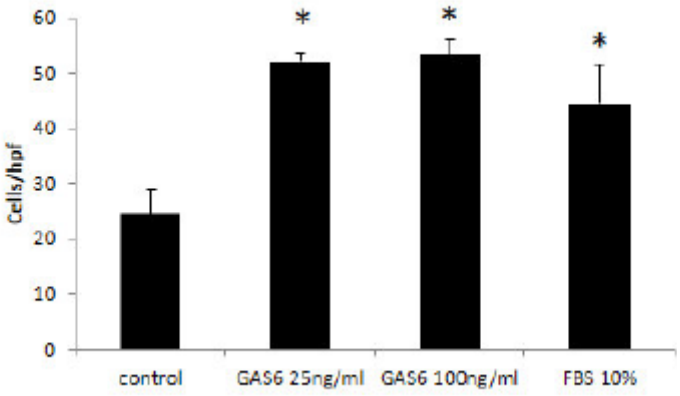


Figure 15. The increase of the cell migration after exposure to Gas6.

We next evaluated the specificity of effects of Gas6 using UNC569, a specific small molecule inhibitor of MERTK. In HSCs, co-treated with UNC5669, the induction of cell migration that we had obtained in response to Gas6 was reverted (*Fig. 16A*).

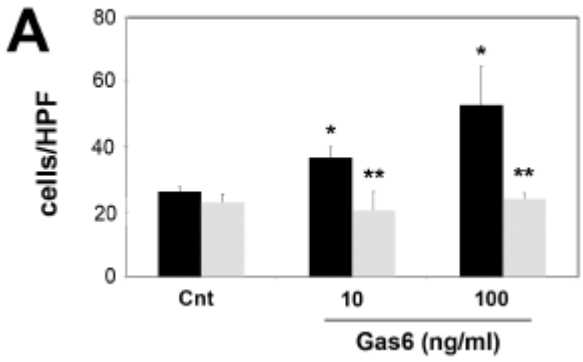


Figure 16A. Induction of cell migration in response to Gas6 (dark columns) and reversion in HSC co-treated with UNC569 (light columns).

The exposure of HSCs to UNC569 resulted also in a significant decreasing in cell viability, indicating that MERTK is involved also in the maintenance of survival of the fibrogenic cells (*Fig.17*B).

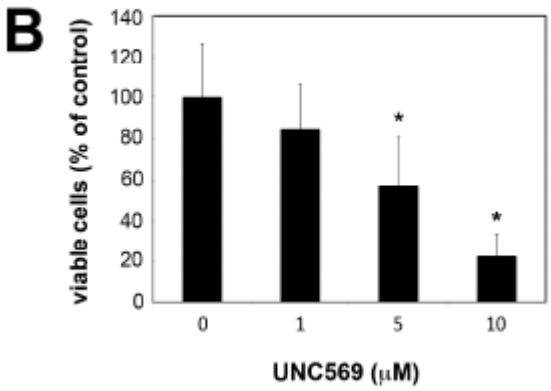


Figure 17B. Exposure of HSC to UNC569 and significant decrease in cell viability.

To exclude that the effects of UNC569 were due to a non-specific inhibition of other molecules, we silenced MERTK using specific siRNA. Also with this approach, the viability of HSCs was significantly reduced, replicating the effects of UNC569 (*Fig.17C*).

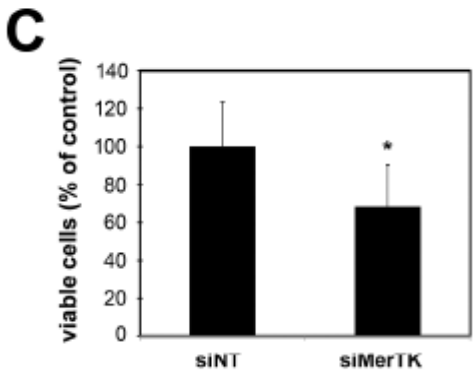


Figure 17C. Silencing of MERTK using specific siRNAs and reduction of HSC viability.

To establish if the reduction of cell viability was due to the program of cell death, HSCs were exposed to increasing concentrations of UNC569. The treatment with UNC569 resulted in the cleavage of PARP and the generation of active caspase-3, compatible with the induction of the apoptosis of HSC. Similar result was obtained in Huh-7 cells, used as a positive control, treated with doxorubicin (*Fig. 17D*).

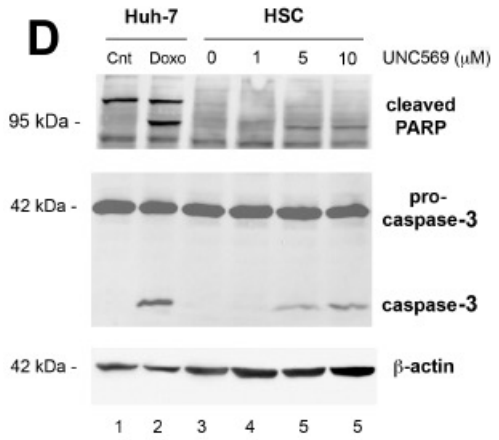


Figure 17D. Cleavage of PARP and generation of active caspase-3 after exposure of HSC and Huh-7 cells respectively to UNC569 and doxorubicin.

Finally, to investigate about the profibrogenic role of MERTK, HSC were stimulated with Gas6 and were assayed for gene expression of procollagen I, in presence or absence of UNC569.

Gas6, slightly but significantly, increased the expression of procollagen I. These effects were inhibited when HSCs were co-exposed to UNC569 indicating that the effects of Gas6 are mediated by MERTK (Fig. 17E).

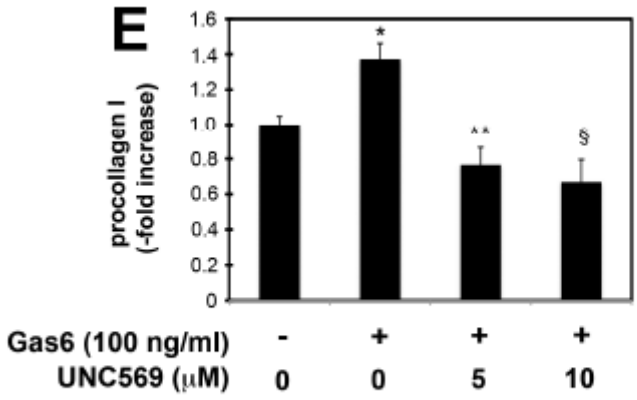


Figure 17E. Procollagen I gene expression in presence or absence of UNC569.

It is clear from these results the crucial role played from MERTK in influencing the development of fibrosisand the progression of chronic liver disease (Petta S, et al., 2015).

The results obtained from the murine models, showed that MerTK is expressed in human HSC and acts directly on this cells by inducing the their activation, by supporting the their survival, by increasing their migration and by inducing the expression of profibrogenic genes, such as procollagene I gene (Petta S, et al., 2015).

In addition, we found that the AA genotypes of MERTK, associated with lower MERTK hepatic expression, is protective against F2-F4 fibrosis in patients NAFLD and is able to condition the progression of this chronic liver disease(Petta S, et al., 2015).

In contrast, the GG genotype has been associated with the fibrotic phenotype in NAFLD, showing a potential direct profibrogenic action.

MerTK is one of the three RTKs of TAM family, is expressed predominantly in M2 cells and acts inhibiting the inflammatory responses and controlling the tissue tolerance (Rothlin CV, et al., 2007; Beherens DK, et al., 2003). In particular, MerTK plays a functional role in the clearance of apoptotic cells and this function is restricted to a subset of M2 macrophages, named M2c, that are characterized by secretion of IL-10 and up-regulation of MerTK. In addition, the M2 differentiation is a key process that regulates inflammation and fibrosis (Smith W, et al., 1998). In chronic disease such as NAFLD, these macrophages play a crucial role to promote the HSCs differentiation in the myofibroblastic phenotype and the fibrogenesis, through MerTK.

Gas6, together with the other ligands, binds MerTK and induces its dimerization and autophosphorylation, activating the downstream signaling (Linger RM, et al., 2008; Braunger J, et al., 1997). Several pathways including PI3-kinase/AKT/Bcl2(survival) and ERK (proliferation). In addition, MerTK suppresses TLR signaling and down-regulates the production of pro-inflammatory cytokines through the phosphorylation of STAT1 that promotes the induction of SOCS-1 and SOCS-3. (Rothlin CV, et al., 2007; Linger RM, et al., 2008).

Normally, MerTK releases factors involved in tissue remodeling, immune responsesuppression and in tumor-promoting(Mantovani A, et al., 2002; Pollard JW, et al., 2004).

However how MerTK is able to conditioning the progression of liver disease remain unknown.

The rs4374383 SNP of MERTK gene is an intronic mutation, that usually creates an alternative splicing site responsible ofthe production of an alternative mature mRNAthat can lead to a splicing variant loss or gain of fuction.

Indeed, the results of this genetic study are not able to givefinal information about the functional mechanism that links MERTK and progression of liver disease especially in neoplastic direction. However, from clinical perspective, it gives a strong suggestion to usethe rs4374383 SNP of MERTK as a genetic biomarker useful to identify the patients with high risk of chronic liver disease progression in order to carry out a more stringent monitoring.

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Curriculum Vitae

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