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POTENTIAL THERAPEUTIC USE OF TRIIODOTHYRONINE (T3) IN HEPATOCELLULAR CARCINOMA

Scientific Disciplinary Code: Med/04

PhD student	**Elisabetta Puliga**
PhD Programme Coordinator	**Prof. Amedeo Columbano**
Tutor	**Prof. Amedeo Columbano**

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To Elisabetta and her wonderful family...

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Abbreviations

- 2-AAF 2-acetylaminofluorene
- AFB1 aflatoxin B1
- AFP α-fetoprotein
- BMI body mass index
- BMP one morphogenetic protein
- CEBPα CCAAT-enhancer-binding protein
- CSCs cancer stem cells
- DENA diethylnitrosamine
- DIO deiodinase
- dsDNA double-stranded DNA
- EGF epidermal growth factor
- ERK extracellular signal-regulated kinase
- ERα estrogen receptor α
- FGF fibroblast growth factors
- G6PC glucose-6-phosphatase
- G6PHD glucose-6-phosphate dehydrogenase
- GGT gamma glutamiltransferasi
- GST-P placental glutathione-S-transferase
- HBV hepatitis B virus
- HBx hepatitis B viral protein
- HCC hepatocellular carcinoma
- HCV hepatitis C virus
- HNF1α hepatocyte nuclear factor 1 alpha
- HNF4α hepatocyte nuclear factor 4 alpha
- IGF-1 insulin-like growth factor
- JNK c-Jun N-terminal kinase
- KLF9 kruppel-like factor 9
- KRT-19 cytokeratin-19

- MAPK mitogen-activated protein kinases
- mTOR mammalian target of rapamycin
- NAFLD nonalcoholic fatty liver disease
- NASH nonalcoholic steatohepatitis
- NCoR nuclear receptor co-repressor
- NF-KB nuclear factor kappa-light-chain-enhancer of activated B cells
- NHR nuclear hormone receptor
- OCT4 octamer-binding transcription factor 4
- OCs oral contraceptives
- PDGF platelet-derived growth factor receptor
- PH partial hepatectomy
- PI3K phosphoinositide 3-kinase
- PPAR peroxisome proliferator-activated receptors
- Prb retinoblastoma protein
- PTEN phosphatase and tensin homolog
- PTU propylthiouracil
- RH resistant hepatocyte model
- ROS reactive oxygen species
- RXR retinoid X receptor
- SMRT silencing mediator of retinoic and thyroid receptor
- STAT signal transducer and activator of transcription
- TACE transcatheter arterial chemoembolization
-T2DM type-2 Diabetes Mellitus
- T3 triiodothyronine
- T4 tyroxine
- TERT Telomerase Reverse Transcriptase
- TGF-β transforming growth factor- β
- TH thyroid hormone
- THR thyrotropin-releasing hormone
- TR thyroid hormone receptor

- TREs thyroid Hormone Response Elements
- TSH thyroid-stimulating hormone
- VEGFR vascular endothelial growth factor
- VDR vitamin D receptors

Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver with a poor prognostic outcome due to limited and ineffective therapeutic strategies. Thus, it is mandatory to develop more efficient and powerful treatments for this aggressive tumor. Recent studies have suggested the possible role of local hypothyroidism in HCC development, in both humans and rodents. Previous observations from our laboratory showed that 1-week exogenous administration of triiodothyronine (T3) induced the regression of preneoplastic hepatic nodules generated by the Resistant Hepatocyte (RH) model of rat hepatocarcinogenesis.

The first aim of this study was to investigate the mechanism/s responsible for the rapid regression of preneoplastic nodules observed 1 week after T3. Transcriptomic analysis showed that a short administration of T3 caused a striking shift of the global expression profile of the aggressive preneoplastic nodules positive for the presumptive progenitor marker cytokeratin-19 (KRT-19) towards that of normal liver or of the indolent KRT-19 negative lesions. This reprogramming preceded the regression of the nodules and was associated with increased mRNA levels of genes involved in the maintenance of differentiated hepatocyte status such as *Hnf4-α* and *Klf9*. These changes were associated with the activation of the T3/Thyroid hormone receptor (TR) axis in KRT-19+ hypothyroid nodules, as shown by a striking increase of *Dio1* mRNA levels.

Unfortunately, the diagnosis of human HCC is too often made at late stages, when there are no effective treatments that would improve patients' survival. Therefore, a second aim of my thesis was to determine whether T3 could exert its anti-tumoral effect also when administered to HCCs bearing rats. Remarkably, we found that 5 cycles of T3 (one week every three weeks) induced the regression of HCCs as observed both at macroscopic as well as at microscopic examination. Indeed, while the liver of all the rats not exposed to T3 displayed multiple HCCs, treatment with T3 reduced the multiplicity of the tumors and the tumor burden. Similar to what was found in preneoplastic nodules, we observed increased expression of genes involved in the maintenance of the hepatocyte differentiated status such as Klf9, Cebp*α* and Hnf4-*α*, paralleled by the loss of neoplastic markers such GST-P and KRT-19.

Furthermore, we also demonstrated the ability of T3 to reactivate the T3/TR axis in fully developed HCCs.

Collectively, these results suggest that T3 acts as a powerful anti-tumoral agent at early and late stages of rat HCC development, in virtue of its differentiating capacity. They also suggest that recently developed thyromimetic drugs, devoid of T3-induced adverse side effects, represent a promising tool in human HCC therapy.

1. INTRODUCTION

1.1 Hepatocellular Carcinoma

Epidemiology

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver.

It is currently the sixth most frequent cancer and the second leading cause of cancer mortality worldwide[1, 2]. In fact, liver cancer is estimated to be responsible for nearly 746,000 deaths in 2012, resulting in a 0,95 mortality/incidence ratio which is the consequence of its poor prognosis and the inefficacy of therapeutic strategies[3]. Globally, the resource-poor countries have the greatest HCC incidence with more than 80% of the estimated 782,000 new cases in 2012.Emerging risk factors such as genetics, *helicobacter pylori* and even microbiota are contributing to HCC diffusion worldwide, including developed countries. [4]

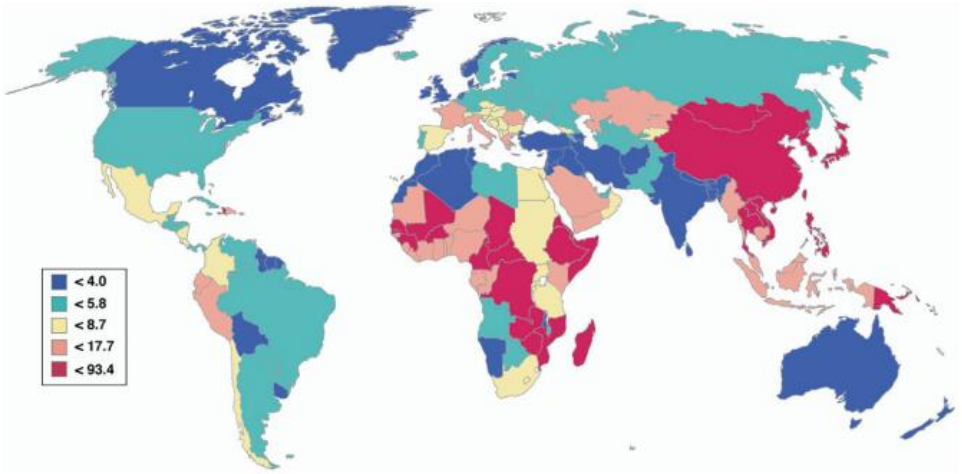

Figure 1. Regional variations in the mortality rates of HCC categorized by age-adjusted mortality rates reported *per* 100,000 persons [5]

Regardless of the significance of the incidence ratio, almost all geographical regions report rates in males that are two- to three-fold higher than rates in females[3] **(Fig.2)**. The gender disparity seems to be associated with the major prevalence of HCC risk factors among males. Indeed, men are more likely to become chronically infected with Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV), and more exposed to alcohol consumption and cigarette smoking. Moreover, the role of sex hormones has been longer investigated, showing that estrogen and upregulation of androgen receptors in tumor tissues may differentially influence the development of HCC in men vs. women. This protective activity of estrogens confers women a resistance to HCC that is hormone-dependent, as displayed by the striking increase of HCC incidence in postmenopausal women. Indeed, women are diagnosed with HCC at a significantly older age than men[5-7].

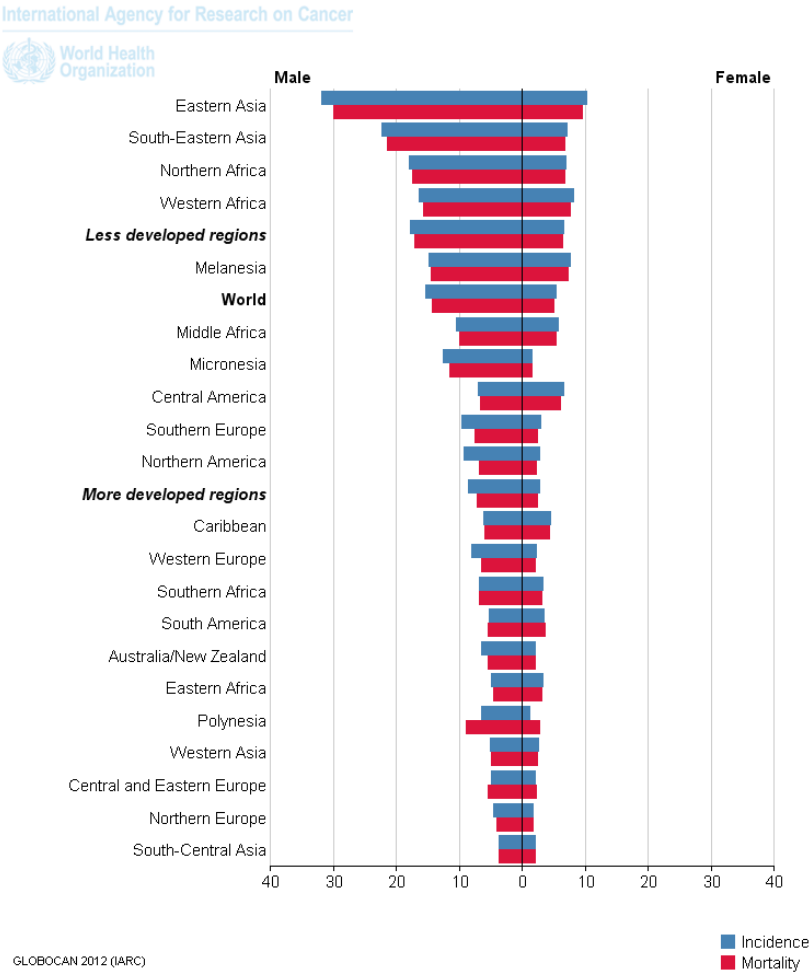

Figure 2. HCC incidence and mortality ratio in male and female [3]

The geographic area together with incidence ratio, sex and etiology display a variable age distribution of HCC[8]. In most countries, the peak of incidence of HCC in females occurs about 5 years later than the peak age group of males. In United States, Canada and United Kingdom, considered low-risk populations, the highest age-specific rates occur among adults aged 75 and older. A comparable pattern is observed among most high-risk Asian populations. On the other hand, while HCC in high-risk male African individuals (Gambia, Mali) exhibits a peak between ages 60 and 65, the peak of females is higher between 65 and 70.These differences are strictly correlated to the distribution of hepatitis virus in the population, the age at viral infection, and the existence of other risk factors [5].

Liver cancer is a multifaceted disease associated with many risk factors and cofactors **(Fig.3)**, and it is characterized by a multistage progression. Indeed, HCC is often preceded by chronic hepatitis, fibrosis and cirrhosis of the liver. Thus, well-known causes of cirrhosis have long been recognized as key risk factors for this cancer. The main predisposing conditions have a variable distribution worldwide.

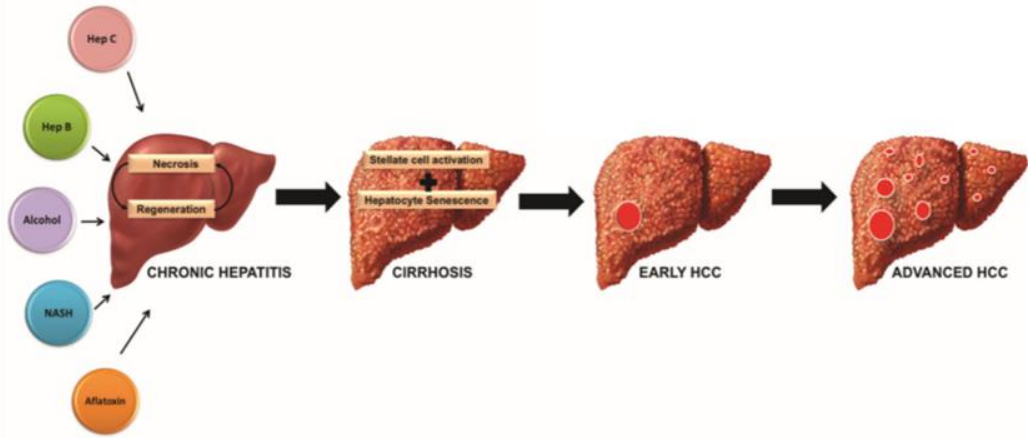

Figure 3. Risk factors involved in the pathogenesis of Hepatocellular Carcinoma[9]

In the most susceptible areas, the leading risk factor is chronic Hepatitis Virus B (HBV) infection. Hepatitis B is transmitted *via* contaminated blood transfusions, intravenous injections, and sexual contacts. In Asiatic regions, the vertical transmission from mother to child is predominant, whereas sibling-to-sibling transmission is more frequent in Africa. On the other hand, chronic hepatitis virus C (HCV) infection represents the predominant risk factor in Japan and Egypt, with lower rates in Europe, United States, and Canada [5, 10, 11].HBV and HCV co-infection in cirrhotic patients increases the risk of HCC with an odds ratio of 165 compared to 17 for hepatitis C and 23 for hepatitis B alone. Moreover, HCC incidence seems to be reduced in patients with a sustained viral response after treatment of HCV with a 54% decrease in all-cause mortality[10, 12].

Other important risk factors for HCC development include toxic exposures (aflatoxins, pesticides, alcohol intake, tobacco), metabolic disorders (non-alcoholic fatty liver disease, diabetes, obesity) and hormonal factors (oral contraceptives). Less common causes include hereditary hemochromatosis, α1-antitrypsin deficiency and autoimmune hepatitis.

1.2 Risk factors

Hepatitis Virus B (HBV) Infection

Chronic hepatitis B is a well-known risk factor associated with the development of HCC as it evidenced by the overlapping between the geographical distribution of HBsAg (Hepatitis B surface antigen) carriers and the occurrence of liver cancer. Epidemiological studies attribute to HBV the 50%– 80% of HCC cases worldwide, while 10%–25% of cases are the result of Hepatitis C Virus (HCV) infection[13]. Most of HBsAg positive patients show cirrhosis of the liver, a key risk factor leading to liver cancer. Consequently, it is necessary to identify new effective antiviral therapies for HBV infection and to encourage the widespread vaccination campaign since the estimated number of patients suffering from virus-related liver disease remains high **(Fig.4)**[14].

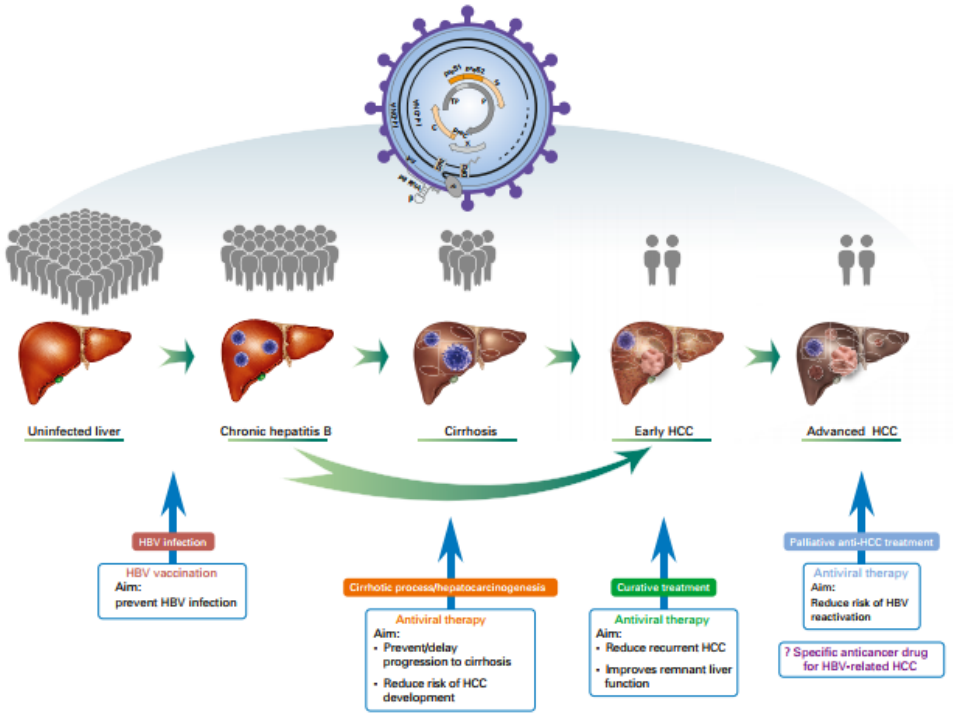

Figure 4. Impact of hepatitis B virus (HBV) infection on the prevention and management of HCC [15]

HBV double-stranded DNA (dsDNA) integration into the nucleus of hepatocytes may lead to the downregulation of tumor suppressor genes or to the modification of sequences of cancer-related genes, including the telomerase reverse transcriptase (TERT). Moreover, a key player in the tumorigenic activity of HBV seems to be the viral protein HBx (Hepatitis Virus B X protein) [15, 16]. Furthermore, genomic methylation and miRNA expression have been recently identified to play a role in the development of HBV-associated HCC[17].

Hepatitis Virus C (HCV) Infection

Globally, around 71 million people are diagnosed with chronic hepatitis C and among them nearly 399 000 die each year from cirrhosis and HCC[18].HCV infection represents the leading cause of HCC in resource-rich countries and it is the first indication for liver transplantation in patients with an established diagnosis of HCC in the U.S[19]. HCV-associated hepatocarcinogenesis is a multistep process involving many phases: establishment of chronic HCV infection, chronic hepatic inflammation, development of liver fibrosis, initiation of neoplastic clones accompanied by irreversible somatic genetic/epigenetic alterations, and progression of the malignant clones. Moreover, HCV core and nonstructural proteins stimulate pro-fibrogenic mediators such as TGF-β and induce p38, MAPK, JNK, ERK, and NF-kB pathways [20]. These observations suggest that the main contribution of HCV to the development of HCC is to promote a cirrhotic tissue microenvironment that serves as a carcinogenic milieu.

Food Contamination by Aflatoxins

Aflatoxins are mycotoxins produced by few Aspergillus species as *A. flavus* and *A. parasiticus*, which are common and largely present in nature. These toxic products usually contaminate crops, including oil seeds, rice, corn, dried fruits and peanuts, that have been stocked in hot, humid and unhealthy conditions. Epidemiological reports and experimental animal models have shown that the whole group of aflatoxins (B1, B2, G1 and G2) are genotoxic, carcinogenic, immunosuppressive substances, and responsible of both acute and chronic toxicity[21]. The aflatoxin B1 (AFB1) is the most potent genotoxic hepatocarcinogen. Once metabolized by the cytochrome P-450 system, AFB1

is converted in the reactive intermediate AFB1-8,9-epoxide. This product is unstable and can form adducts with the DNA (AFB1-guanine adducts) leading to genetic changes in liver cells.

Pesticides

Pesticides exposure is thought to be an environmental factor implicated to increase the risk of HCC. Several mechanisms are thought to contribute to pesticides carcinogenic potential. Spontaneous initiation of genetic changes, cytotoxicity with persistent cell proliferation, oxidative stress, inhibition of apoptosis and suppression of intracellular communication were observed after exposure[22, 23]. A case-control study of HCC in HBV and/or HCV infected patients from Egypt suggested that pesticides had an additive effect on the risk of HCC in rural males, amongst whom the use of carbamate and organophosphate compounds is very common[23].

Alcohol

Heavy alcohol consumption is a well-known HCC risk factor leading to liver damage through endotoxins, oxidative stress, and inflammation[24, 25]. Analysis of different geographical areas, showed that alcohol intake can act as a primary cause or as a cofactor for HCC onset. In poor-resources countries alcohol acts as cofactor, since its consumption is accompanied by other risk factors, such as HBV and HCV virus or diabetes. The mechanism by which alcohol causes HCC is still unclear but it is hypothesized that oxidative stress, altered retinoic acid metabolism, DNA methylation, genetic susceptibility and decreased immune surveillance may play an important role in tumor development[26].

Tobacco smoke

Cigarette smoking is associated with HCC development, independently of geography and race-ethnicity [27-29]. Cigarettes carry over 4000 toxic substances which are considered dangerous and toxic for almost every organ in the body. Liver is the main site for the metabolism and transformation of more than 40 tobacco-related active compounds. Several components of cigarettes are known liver carcinogens in humans and animal models such as 4-

aminobiphenyl, arsenic and vinyl chloride [28-30]. However, the effect of cigarette smoking on HCC biology remains still elusive.

Metabolic disorders

Numerous epidemiological studies performed mainly in the United States, revealed that a conspicuous number of HCC cases are not related to the most common risk factors (chronic infection by HBV, HCV, alcohol consumption). In general, approximately 15–50% of HCC cases remain idiopathic, suggesting that other risk factors are responsible for this increase[31].Among these patients, a large proportion includes patients with metabolic disorders, such as nonalcoholic fatty liver disease, obesity and diabetes[32].

Nonalcoholic fatty liver disease (NAFLD)

NAFLD is the most common chronic liver disease in the United States and represents an increasingly important etiology of HCC[33]. The development of HCC in NAFLD is most likely multifactorial and involves obesity-mediated mechanisms including low-grade chronic inflammatory response, increased lipid storage and lipotoxicity, alteration of gut microbiota with increased levels of LPS (lipopolysaccharide) and insulin resistance with hyperinsulinemia and increased IGF levels[34]. This association is frightening, due to the globally high prevalence of these conditions and may contribute to the rising incidence of HCC observed in many industrialized countries.

Obesity

In 2003, Calle and colleagues[35], reported that 14% of all cancer deaths in women and 20% in men in US occurred in obese patients. In this study, the authors correlated the relative risk (RR) of cancer of over 900,000 individuals with their body mass index (BMI). Matched with normal weight individuals, patients with a BMI greater than 35 exhibited an increased RR of dying of liver cancer. Subsequent studies from Asia and Europe supported a key role for obesity in HCC risk, either alone or as a cofactor[34, 36].

Diabetes

Accumulating epidemiological evidence indicates that type 2 diabetes mellitus (T2DM) promotes the development and progression of HCC. Davila *et al*.[32], evaluated that the 14% of the United States population affected by diabetes had a three-fold risk to develop HCC. The association between diabetes and HCC has been further demonstrated by studies focused on populations from different geographical locations indicating that in most individuals, diabetes may exist for a long time before the diagnosis of HCC [37, 38].

Hormonal factors

There is biological and experimental evidence for a possible role of oral contraceptives (OC) in liver neoplasia. Indeed, nuclear estrogen receptors are present in hepatocytes and are increased in HCC, suggesting a hormonal responsiveness of hepatic neoplastic tissue[39].Estrogens are thought to cause liver neoplasia by increasing proliferation rates and by inducing spontaneous mutations. The estrogen and progesterone components of OC have been shown to induce and promote liver tumors in animals [40]. Moreover, several human studies have reported an increased risk of developing benign liver tumors, such as hepatic cell adenoma and focal nodular hyperplasia in women using OC.

1.3 Natural history of HCC

Hepatocarcinogenesis is a multistep process that arises from an altered hepatic microenvironment, usually related to chronic liver disease, characterized by massive inflammation and fibrosis. What seems particularly significant in liver oncogenesis in humans is the window of time from the beginning of the inflammatory process or the exposure to risk factors and the occurrence of HCC. It has been shown that in HCV-patients, HCC unfolds during a progression that may take more than 30 years after the chronic infection diagnosis [41, 42]. Conversely, the time course of HBV-related carcinogenesis is less predictable since HCC may precede the occurrence of cirrhosis, in particular with chronic HBV infection in endemic areas [43].

Trevisani and colleagues [44], suggested that the natural history of HCC can be divided into three distinct phases:

- molecular;
- preclinical;
- clinical or symptomatic **(Fig.5)**.

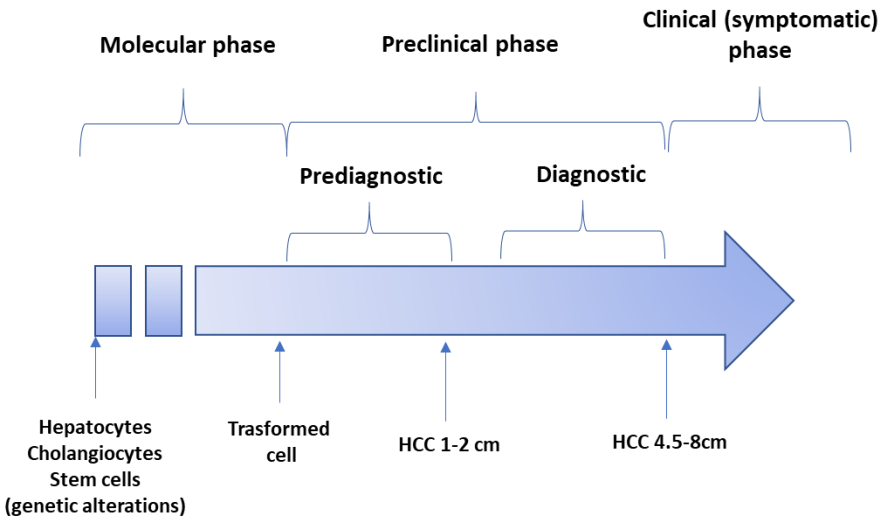

Figure 5. The natural history of HCC can be divided into three distinct phases: molecular, preclinical and clinical or symptomatic [44]

The molecular phase includes the upregulation of mitogenic pathways and sequential genomic alterations leading to hepatocyte transformation. Gradually,

the production of monoclonal populations of aberrant and dysplastic hepatocytes, characterized by telomere erosion and telomerase re-expression, microsatellite instability, and occasionally structural aberrations in genes and chromosomes takes place. Mechanisms of cellular transformation are different depending on the cell type. Genetic alterations involving differentiated cells (hepatocytes and cholangiocytes) are thought to confer a growth advantage by promoting proliferation and inhibiting apoptosis, whereas those involving stem cells interfere with the differentiation process. The malignant hepatocyte phenotype may be produced by the disruption of many genes that function in different regulatory pathways, producing several molecular variants of hepatocellular carcinoma [44].

The preclinical phase consists in an initial period, in which focal areas of abnormal, immature hepatocytes and dysplastic foci (<1 mm) are considered too small to be detected by imaging techniques. On the contrary, during the 'preclinical diagnostic' phase, the tumor is detectable because of its burden but still asymptomatic and lacking invasive pattern: macrovascular invasion or extrahepatic spread [44, 45].

Finally, the clinical or symptomatic phase takes place with the occurrence of symptoms caused by the tumor burden and the severe impairment of liver function. In patients with chronic liver disease, HCC usually becomes symptomatic when it reaches 4.5–8 cm [44, 46].

Unfortunately, the diagnosis of HCC is too often made during the symptomatic phase and at this late stage, there is virtually no effective treatment that would improve the survival. Additionally, the morbidity related to therapy is too high. Moreover, patients with advanced-HCC have a survival rate of only 0%–10% [47]. In contrast, patients with an early diagnosis of HCC can achieve five-year survival rates of over 50% [48]. With an appropriate screening and surveillance, many patients can and should be diagnosed with early disease and preserved liver function. Indeed, patients who are diagnosed early have various treatment options leading to improved outcomes. Given the well-known correlation between liver cirrhosis and HCC development, there is a strong rationale for surveillance of patients with cirrhosis regardless of the etiology [49].

Prevention of HCC is a crucial aim that can be achieved with the development of preventive procedures. A successful example of the preventive intervention was validated by the vaccination program against HBV which was realized in the 1980s and efficaciously reduced both the number of HBV carriers and the incidence of HCC in children. Moreover, interferon therapy in patients with HCV, with and without cirrhosis, is associated with a lower risk for developing HCC [50-52].

The serum marker α-fetoprotein (AFP) and ultrasounds represent the most relevant diagnostic tests for HCC; however, levels of sensitivity and specificity of these screenings are very limited when used alone [53, 54]. Indeed, AFP is not elevated in all patients with HCC. Factors such as age, sex, infection with HBV and HCV, cirrhosis and acute liver necrosis, especially size and form of tumor pathology can influence AFP level [55].

Generally, therapeutic approaches for the treatment of HCC can be classified into three categories: potentially curative, palliative, and symptomatic. Potentially curative treatments, including liver resection, transplantation, and local ablation, are associated with promising 5-year survival rates of up to 75% [56].On the other hand, because of an insufficient number of donors, advanced tumor stage, or liver dysfunction, less than 20% HCC patients are eligible for such treatments [32, 57].

Most HCC patients are subjected to palliative or symptomatic treatment. The 3-year survival rate for palliative treatment is 10–40%, and the duration of survival for patients who receive symptomatic treatment is < 3 months [58].

Among palliative cares, the most commonly used for loco-regional HCC as well as for down-staging tumors is the trans arterial chemoembolization (TACE). The rationale for using TACE is the neoangiogenic property of HCC and its mechanism of action on the hepatic arterial supply of the tumor. Embolization of the hepatic artery branch leads to selective tumor hypoxia and eventually tumor necrosis [59].

The pathophysiologic complexity of HCC has made medical treatment particularly challenging. Indeed, it has been problematic to provide a suitable tumor therapy able to maintain the liver function.

In 2005, the FDA approved Sorafenib for the treatment of unresectable HCC and recommended its use as first-line therapy for HCC patients who cannot benefit from therapies that are potentially more effective, such as TACE or local ablative therapy [48].

Sorafenib is an oral multikinase inhibitor that suppresses tumor growth and angiogenesis by inhibiting the Raf/MEK/ERK signaling pathway and receptor tyrosine kinases, such as vascular endothelial growth factor receptor (VEGFR) 1, VEGFR-2, VEGFR-3, and platelet-derived growth factor receptor beta (PDGF-β) [60]. In the pivotal Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial, sorafenib-treated patients had a median time to progression of 5.5 months and survival of 10.7 months, a 2.8-month improvement in survival over placebo [61].

Although, Sorafenib is a unique neoangiogenic targeting agent with encouraging results, the benefits obtained from this treatment are still disappointing and, thus, it is mandatory to find alternative effective treatments. Further studies are needed to identify target pathways and biomarkers to improve survival and tumor regression. Most patients diagnosed with HCC have advanced disease, and these patients represent the main priority for the progress of effective therapies. Advanced HCC remains a crucial medical necessity for which available research resources should be prioritized.

Moreover, detailed analysis and characterization of the early molecular mechanisms driving the development of HCC would improve both diagnosis and treatment of this cancer. Future clinical trials could identify additional agents and cares for a personalized therapy for the HCC community and furthermore the development of tissue, serum, and other validated biomarkers would help to screen those patients who will benefit most from emerging treatment options.

1.4 Animal models of Hepatocarcinogenesis

The Resistant Hepatocyte model (RH)

Several rodent models have been used in defining the pathogenesis of HCC and have contributed to the current knowledge of HCC. Indeed, because of its multistage nature, the molecular pathogenesis of this cancer cannot be completely understood without more information on the molecular alterations characterizing its early development. Unfortunately, the study of the early steps of HCC development in humans is complicated due to the late stage at which the tumor is diagnosed and to the heterogeneity inside a tumor that makes difficult a clinical classification. In this scenario, animal models assume a crucial role as they are essential for the understanding of the molecular/metabolic alterations taking place at the different stages of HCC development.

Among the most widely used experimental models for the characterization of hepatocarcinogenesis, we employed the Resistant Hepatocyte (RH) rat model, also known as Solt & Farber model, which allows to carry out the analysis of the different sequential steps leading to HCC development [62].

In this protocol, initiation is achieved through a single necrogenic administration of a chemical carcinogen, diethylnitrosamine (DENA), followed by a promoting regimen consisting of a 2 week-diet supplemented with the selective inhibitor of hepatocyte proliferation, 2-acetylaminofluorene (2-AAF), coupled with a powerful growth stimulus, such as 70% partial hepatectomy (PH). Initiated cells undergo clonal expansion giving rise to early preneoplastic lesions, immunohistochemically identified by their positivity for the placental form of the enzyme glutathione-S transferase (GST-P), gamma-glutamyl transpeptidase (GGT), glucose-6-phosphate dehydrogenase (G6PDH), and several others [62-64].

In the following weeks, foci progress to a nodular stage becoming macroscopically visible and occupying most of the liver. During the carcinogenic process, a slow but continuous regression of the majority of the preneoplastic lesions occurs through a remodeling process in which hepatocytes gradually

lose the staining for GST-P and other preneoplastic markers, and reacquire a differentiated phenotype.

Conversely, only a smaller fraction of preneoplastic nodules progresses to develop early HCC (eHCC) and 12-14 months after DENA, to advanced HCCs (aHCC).

Interestingly, GST-P+ preneoplastic nodules can be further divided into Cytokeratin-19 (KRT-19) positive or negative lesions, depending on the expression of this protein. KRT-19 is a protein of the cytoskeleton intermediate filaments, normally expressed in adult liver by the bile duct epithelial cells, but not by normal hepatocytes [65].

Previous studies from our laboratory, showed that almost all the HCCs arising in this model are KRT-19+, although only a minority of preneoplastic lesions developed at early stages of the process are positive for this marker. Furthermore, HCCs and KRT-19+ nodules share a common gene expression profile, suggesting that rat HCC derives from a sub-population of KRT-19+ preneoplastic lesions.

The translational value of the Resistant Hepatocyte model has already been demonstrated; indeed, comparative genomic studies on laser capture-micro dissected early lesions showed that the HCCs generated with the RH model have a gene expression signature very similar to a specific human HCC subtype that expresses KRT-19 and is characterized by the worst prognosis[66].

Moreover, the finding that 78% of genes and 57% of miRNAs deregulated in rat HCC are similarly altered in human HCC and that 76% of these genes are already deregulated at very early stage of the process, further supports the translational value of this model in predicting not only the molecular changes relevant to human HCC, but also the stage at which these changes occur [67].

1.5 Thyroid Hormones (THs)

Thyroid hormones, L-thyroxine (T4) and L-triiodothyronine (T3) are secreted by the follicular cells of the thyroid gland. The release of these hormones is regulated by the thyrotropin-releasing hormone (THR) together with the hypothalamus and thyroid stimulating hormone (TSH) from the anterior pituitary gland via negative feedback loop [68]. Physiologically, T4 is the principal hormone secreted in the blood stream, but the prevalent active form is represented by T3. The main pathway leading to the conversion of T4 in T3 occurs in peripheral organs by the selenoenzymes iodothyronine deiodinase I and II (Dio1 and Dio2) [69]. Indeed, Dio1 is responsible for the switch of the majority of T4 in T3 in liver and kidney through 5'-deiodination of the outer ring of T4. Dio2, found in pituitary, brain and brown adipose tissue, principally converts T4 to T3 for intracellular use **(Fig.6)**. Moreover, these two enzymes lead to the generation of rT3 by 5-deiodination, a crucial step for thyroid hormones inactivation. Dio3 prevents the excess of T3 as well depleting sources of active hormone by inner ring deiodination [70, 71]. rT3 and T3 can be further deiodinated in the liver and sulfo- and glucuronide-conjugated before excretion in the bile [72, 73].An enterohepatic circulation of TH is represented by the intestinal flora that deconjugates some of these compounds and promotes the reuptake of TH.

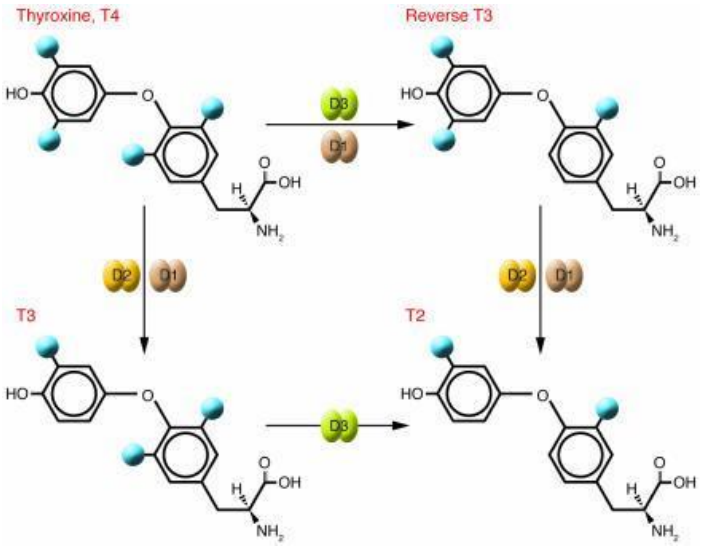

Figure 6. Basic deiodinase reactions [71]

Thyroid hormones (THs) play critical roles in many physiological processes such as differentiation, growth, and metabolism. TH is required for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate. Although it has been postulated the existence of rapid non-genomic mechanisms [74], the best known effects of these hormones require the presence of the thyroid hormone nuclear receptors (TRs) [75]. Indeed, TRs belong to the nuclear receptor superfamily that includes the estrogen receptor, vitamin D receptor, peroxisome proliferator-activated receptors (PPARs), retinoic acid receptor (RAR), retinoid X receptor (RXR). These receptors function as modulators of gene expression by virtue of their ability to recognize specific DNA sequences [76, 77]. TRs consist in a single peptide that is folded into three modular functional domains: an *amino-terminal domain* (A/B domain), a central *DNA-binding domain* (DBD) and a carboxyl-terminal *ligand-binding domain* (LBD) [78] **(Fig.7)**.

The *amino-terminal domain* shows a variable length and divergent sequence among the TR isoforms. This region is a target of a variety of phosphorylation events that influence the receptor function [79]. Previous studies suggested its involvement in cell-specific and promoter-specific transcription [80]. Moreover, the A/B domain seems to recruit an assortment of co-regulatory proteins that can participate in ligand-independent transcriptional regulation [81]. Tian and colleagues showed that A/B domain influences ligand-dependent recruitment of coactivators to the ligand-binding domain [82].

The *DNA-binding domain* is in the central portion of the receptor and presents two zinc fingers, each composed of four cysteines coordinated with a zinc ion. This domain integrity is crucial for the correct DNA-binding and transcriptional activity of the TR [83]. DBD is essential for the sequence-specific recognition of hormone response elements. Indeed, within the DBD, there are dimerization interfaces and contact points for the interaction with co-receptors important to stabilize the DNA binding [75].

The "*hinge region*" is a interdomain that joins together the DNA-binding domain and the ligand-binding domain. This flexible linker contains key nuclear localization motifs and can contribute to the recruitment of several regulatory

proteins, either alone or in conjunction with the other nuclear receptor domains[84].

The *ligand-domain* is the region responsible for the receptor-hormone interaction and it also plays critical roles for dimerization, transactivation, and basal repression by unliganded TR. In fact, LDB consists of 12-helices which undergo key conformational changes upon ligand binding from a more open conformation to a closed one, which has been associated to a “mouse trap” mechanism.

In the absence of thyroid hormone, this surface task can interact with helical motifs characterizing SMRT and NCoR family of corepressors, resulting in the enrollment of these corepressors.

The corepressors, in turn, recruit deacetylases and additional histone modifiers that, by altering the chromatin template, lead to repression of transcription [85, 86].

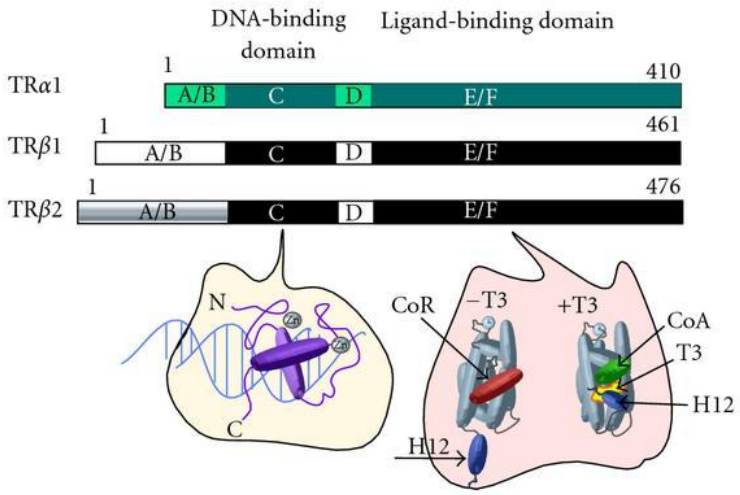

Figure 7. Domain comparison of different TR isoforms and schematic representation of DNA- and ligand-binding domain crystal structures [87].

Two thyroid hormone receptor genes, THRα (NR1A1) and THRβ (NR1A2), have been identified on human chromosomal regions 17q11.2 and 3p24.3, and in 10q31 and 15p16 rat chromosomal regions [88, 89]. The alternative splicing of primary mRNAs contributes to generate additional receptor diversity. In mammals, THRα encodes three C-terminal variants: α1 (NR1A1a), α2

(NR1A1b) and α3 (NR1A1c). TRα1 binds T3 and DNA representing a fully functional receptor, while TRα2 and TRα3 do not bind T3 and are weak dominant negative antagonists *in vitro*, although their roles *in vivo* are still unclear [88-90]. On the other hand, THRβ encodes two N-terminal variants, β1 (NR1A2a) and β2 (NR1A2b), which are transcribed from distinct promoters. The β1 N terminus is encoded by two exons that are replaced by a single exon in β2 [91-94]. TR mRNAs are widely expressed, but there are differences in concentrations of the isoforms in individual tissues (**Table 1**) and in different developmental stages. TRα1 is constitutively expressed during embryonic development while TRβ is expressed toward the later stage of development [95].

Table 1. Expression of THR isoforms in different tissues

THR ISOFORM	LIVER	KIDNEY	HEART	BRAIN	THYROID	RETINA	LUNGS	SKELETAL MUSCLES	REFERENCE
THRα1	+	+	+	++	-	-	+	+	94
THRα2	+	+	+	++	-	-	+	+	94
THRβ1	++	++	++	++	++	-	+	+	77, 94
THRβ2	-	-	+	++	-	++	+	-	94, 91
THRβ3	++	++	+	+	-	-	++	+	94

Independently from their localization, TRα and TRβ exert their action on gene expression through direct interaction with specific DNA sequences known as thyroid hormone response elements (TREs), usually found in the 5'- flanking regions of T3-responsive genes. TREs usually consist of two or more tandem repeats of AGGT(C/A) sequences separated by four base pairs (direct repeat 4, DR4), even if TREs can exist as palindromes or inverted palindromes [96]. The TRs can bind to DNA as monomers, homodimers, or heterodimers with other nuclear receptors, including retinoid X receptor (RXR) and other retinoic acid receptor subtypes, and vitamin D receptors (VDR) [97, 98]. TRs generally heterodimerize with RXR; this heterodimer exhibits the highest T3 binding affinity and remains stable during ligand binding [97, 99].

Furthermore, these receptors can act in a ligand-independent manner modulating the transcription rate of target genes depending on whether or not the TR is bound to T3[78].

In the absence of T3, a positively regulated target gene will have a TRE to which the TR binds and recruits a co-repressor, such as N-CoR or NCoR2. The co-repressor interacts with histone deacetylases, which modify the chromatin structure resulting in decreased gene transcription [100].In the presence of T3, the repressive complex is not stable, the co-repressors are released and the recruitment of coactivators, such as SRC-1 occurs **(Fig.8)**[101-103].

Co-activators induce remodeling of chromatin by acetylating or methylating histones or altering the DNA conformation, which changes the interactions among RNA polymerase and other transcriptional factors [104].

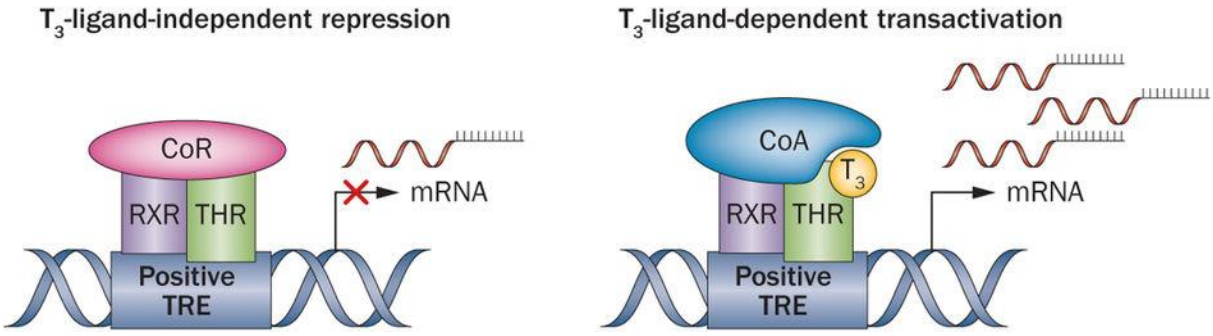

Figure 8. Model of gene regulation by thyroid hormones. Abbreviations: CoA, coactivator; CoR, co-repressor; RXR, retinoid X receptor; THR, thyroid hormone receptor; TRE, thyroid-hormone responsive element [78]

1.6 Thyroid Hormone mimetics

The administration of exogenous thyroid hormones has been widely used as replacement therapy to correct hypothyroidism in patients with low levels of these hormones [105].Moreover, other beneficial effects related to thyroid hormones administration include reductions in serum LDL–cholesterol and body fat[106, 107].

Since negative side effects such as tachycardia and heart failure, muscle wasting, and other symptoms including fatigue and anxiety, preclude thyroid hormones therapeutic use to obtain beneficial effects, scientists have long been intrigued by the possibility to develop thyroid hormone derivatives[108-111].

In the last decades, a deeper understanding of TRs structure and function together with a deeper knowledge of thyroid hormone analogue chemistry have led to the synthesis of potent thyromimetics with receptor subtype-selective activities[109, 112]. **(Fig. 9)**

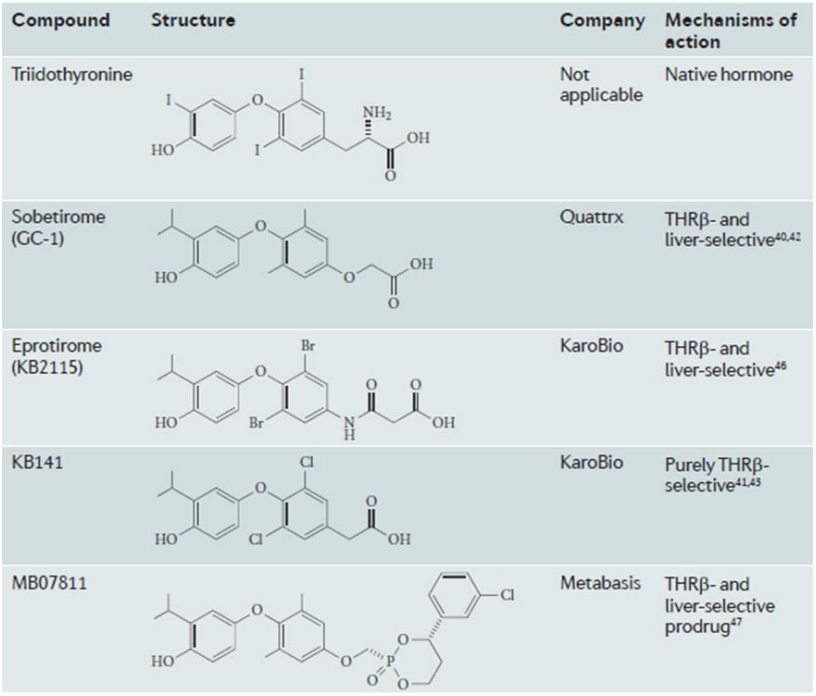

Figure 9. Structures of thyroid hormone mimetics [105].

After the first efforts on the development of selective thyroid hormone receptor modulators, cloning of the thyroid receptor led to the identification of two major isoforms with different tissue distributions throughout the body. The TRα isoform is predominantly present in the brain, heart, and skeletal muscles, whereas TRβ is mainly localized in the liver and also in the brain[113]

Studies of the phenotypes of knockout mice for the TR genes have confirmed that TRα is involved in the control of the heart rate, and showed that TRβ plays a major role in the regulation of serum cholesterol levels and in the feedback inhibition of thyroid hormone production[114, 115]. These observations suggest that selective activation of TRβ would promote the beneficial effects of thyroid hormone, while avoiding deleterious effects on the heart.

One of the first-generation TRβ agonist is GC-1 also known as Sobetirome. Designed at the University of California San Francisco, GC-1 is a scaffold compound that can be synthesized efficiently and is more easily modified than native thyroid hormone[111]. In cell culture assays testing the hormone binding and activity, it was shown to be approximately five times more selective for TRβ1 than for TRα1, and able to bind all major TRβ isoforms with similar affinity to T3.

Sobetirome preferentially accumulates in the liver while the uptake in other tissues, including heart and skeletal muscle, is low relative to the uptake of T3.

A structure–activity analysis of thyroid hormone derivatives identified another TRβ1 agonist, KB141 [116]. This thyromimetic displayed an affinity for TRβ 15 times higher than its affinity for TRα as determined by *in vitro* binding and activity assays. KB 141, can induce weight loss and reduce cholesterol and lipoprotein (a) with no effect on heart rate [117, 118].Contrary to GC-1, it does not present a preferential uptake in the liver.

The reasons why different thyromimetics show tissue distributions that diverge from native thyroid hormones is not clear, but are probably related to high rates of liver first-pass uptake, and differences in cellular uptake and retention mechanisms. These findings suggest that differences in tissue uptake could contribute to GC-1-selective actions. Within target cells, such as hepatocytes, GC-1 will interact selectively with TRβ rather than TRα. It is thought that GC-1,

may also display gene-specific actions relative to T3, but the extent of this effect and its contributions to the actions of the drug are still unknown[119, 120].

More recently, there was optimism about the synthesis of a second generation of highly selective TRβ agonists characterized by additional tissue-specific effects.

Among them, KB2115, commercially known as Eprotirome, is a TRβ-selective ligand that is preferentially taken up by the liver and lacks extrahepatic side effects. Despite a promising activity on LDL, cholesterol and triglycerides reduction together with no potentially deleterious cardiac or bone effects, cartilage damage in long-term dog models led to the withdrawal of this thyromimetic compound from clinical trials [121]

Finally, to improve the tissue-specificity of these thyromimetics avoiding extrahepatic flow, many research groups aimed to design pro-drugs able to exert their thyromimetic effect only following tissue-selective activation by enzymatic cleavage [122].

In this respect, MB07811 is a liver-selective prodrug, that in its active form, MB07344, is strongly TRβ-selective (>tenfold relative to TRα) and binds TRs with significantly lower affinity than other selective analogues.

Thus, actions of MB07811 could be related to a high liver selectivity, TRβ selectivity or a combination of both. Moreover, although most differences between MB07811 and other TRβ-selective compounds probably result from liver selectivity, its lower TR affinity might also contribute to reductions in both efficacy and side effects relative to other ligands.

1.7 The proliferative and anti-tumorigenic effect of T3

Liver is the main target organ of thyroid hormone action and it has been shown that approximately 8% of the hepatic genes are regulated by thyroid hormone *in vivo* [123].In addition to the central role in deiodination to activate and deactivate thyroid hormones, liver performs specific functions related to thyroid hormone transport and metabolism [124]. Consequently, the impact of THs signaling on liver hepatocytes has been largely examined.

Triiodothyronine influence on liver growth has been demonstrated by several studies showing that T3 administration promotes liver regenerative capacity after partial hepatectomy (PH) [124-126]. This role has been supported by the observation of a decrease in the normal hepatic regeneration response after partial hepatectomy [127], in thyroidectomized rats. On the other hand, exogenous administration of T3 triggers a wave of hepatocyte proliferation that resembles in timing and magnitude of DNA synthesis that induced by 40% hepatic resection [124, 128, 129].

At a transcriptional level, Pibiri *et al*. [130] showed an earlier expression of cyclin D1 in rats administered with T3 compared with rats subjected to PH, supporting the hypothesis that hepatocyte proliferation induced by ligands of nuclear receptors occurs through mechanisms clearly distinct from those observed in liver regeneration after PH or liver cell necrosis [131]. Furthermore, previous studies by Alisi *et al*. [132], suggested that rats with experimental hyperthyroidism showed an increased hepatic expression of cyclins D1, E, and A as well as elevated Cyclin-dependent kinase (Cdk) activity and reduced Cdk inhibitor expression. On the other hand, in the same study it has been shown that propylthiouracil (PTU)-induced hypothyroidism caused a decrease in cyclin D1 expression and Cdk activity.

β-catenin, an important nuclear effector of the Wnt signalling pathway, has also been described as responsible for T3-induced mitogenesis in liver[133]. Indeed, while in wild-type mice T3 treatment induces a robust wave of hepatocyte proliferation, no mitogenic response occurred in the hepatocyte-specific β-catenin knockout mice [134]. Even though the molecular mechanisms through which T3 stimulates hepatocyte proliferation are still unclear, it is noteworthy

that its mitogenic effect occurs in the absence of activation of transcription factors such as NF-κB, STAT3 or AP-1, and it is not associated with an increased expression of *c-fos*, *c-jun* or *c-myc* proto-oncogenes [130].

In spite of the evidence that T3 stimulates mitosis in hepatocytes of normal liver, T3 treatment reduces the number and size of GST-P+ preneoplastic nodules and decreases the incidence of HCC development and lung metastases [135] in rats treated with diethylnitrosamine (DENA) and exposed to the Resistant Hepatocyte model. Therefore, T3 seems to have a dual and conflicting effect, i.e the capability to determine the regression of pre- and neoplastic lesions while retaining its mitogenic effect.

Ledda-Columbano and colleagues[135], shed light on this issue by demonstrating the ability of T3 to induce a re-differentiation program. Accordingly, Perra *et al*. showed, in a nutritional model of HCC, that administration of either T3 or the TRβ agonist GC-1 promotes a striking reduction in the number of preneoplastic liver lesions accompanied by the reacquisition of the activity of glucose 6-phosphatase and adenosine triphosphate, two enzymes expressed in normal hepatocytes [136].

Recently, a significant reduction in tumor burden, associated with decreased tumor cell proliferation has been achieved by GC-1 treatment in FVB mice bearing HCCs generated by hydrodynamic tail vein injection of hMet-S45Y-β-catenin plasmid, using the sleeping beauty transposon-transposase. The reduced expression of p-Met (Y1234/1235), p-ERK and p-STAT3 seen after the administration of GC-1, suggests that this thyromimetic exerts a notable antitumoral effect on hMet-S45Y-β-catenin HCC, by inactivating Met signaling [137].

In vitro studies published by Yen et al. [138], demonstrated that the growth of hepatoma cell line overexpressing thyroid receptors TRα1 or TRβ1 (HepG2-TR) was inhibited by over 50% following treatment with T3. According to the authors of this study T3 can stimulate TGF-β promoter activity, leading to a suppression of Cdk2, cyclin E and phospho-retinoblastoma (pRb) protein expression. Additionally, Lin *et al*.[139], performed cDNA microarray analysis to study the mechanism of cell proliferation inhibition induced by T3 treatment in HepG2-TR

cells. Genes differentially expressed following T3 treatment, including ProT and STAT, were further studied. Quantitative-reverse transcription polymerase chain reaction revealed that ProT and STAT mRNA levels, were down-regulated 2.2- or 2-fold respectively after T3 treatment. Moreover, the analysis of the mRNA or protein level of major cell cycle regulators revealed a 2-4-fold upregulation of P21 while Cdk2 and cyclin E were down-regulated 2 to 4 folds following the treatment.

These results provide further evidence for the growth inhibition effect of theT3/TR axis on HepG2-TR cells.

1.8 Thyroid hormone levels and cancer

To date, conflicting results are reported in the literature about the role of THs in cancer development. Recent studies reported that subclinical hyperthyroidism might increase the risk of certain solid tumors [140]. In a population-based case–control study, hyperthyroidism was identified as a significant ovarian cancer risk factor [141].

Moreover, patients with a history of hyperthyroidism exhibit a two fold increase in pancreatic cancer risk [142]. For prostate cancer, men with low TSH levels suggestive of a hyperthyroid function were at higher risk compared with men showing normal levels of the hormone[143]. On the contrary, Pinter *et al*. [144] associated elevated TSH levels with the development of liver tumors of bigger size compared with patients with low TSH levels. In the same study, the authors showed that patients with elevated fT4 more frequently had elevated C-reactive Protein levels which indicate worse prognosis in HCC.

However, several clinical studies reported that also hypothyroidism can be involved in the development of different tumors. In fact, a condition of hypothyroidism has been shown to be frequently found in cancer patients and to be associated with poor response to therapy [145]. Despite these evidence Cristofanilli and colleagues [146],showed a lower rate of primary breast carcinoma and a reduced risk of developing invasive disease in hypothyroid patients. Moreover, hypothyroidism seems to be clinically favorable in patients

with glioblastoma multiforme, since treatment with the anti-thyroid medication propylthiouracil in combination with tamoxifen appears to increase the overall survival [147].

Furthermore, it has been reported that hypothyroidism might be a possible risk factor for liver cancer in humans [148]. Indeed, hypothyroidism plays an important role in metabolism and can lead to different conditions which either directly constitute an HCC risk factor or can contribute to the development of known predisposing conditions for HCC, such as obesity [149-152], diabetes [153-156] and non-alcoholic fatty liver disease [153, 157].

Hassan *et al*. [158], reported an association between hypothyroidism and HCC risk in men and women in a case-control study. Independently of established HCC risk factors, a long-term history of hypothyroidism was correlated with a statistically significant higher risk of HCC development in women. According to these findings Reddy *et al*.[148], demonstrated that hypothyroidism was more frequent in HCC patients with an unknown etiology than in patients with alcoholic liver disease or HCV, suggesting that hypothyroidism may be a permissive factor for the development of HCC.

1.9 TRs and HCC

Since THs activity is modulated by their interaction with the TRs, several studies have been performed to examine the role of these receptors in HCC development and progression [159].The first observation relating TRs with the tumorigenic process came from the demonstration that *v-ErbA* oncogene, isolated from an avian retrovirus, is an altered form of the *TRα* gene [160] that antagonizes TRs activity by competing for TREs or co-activator binding factors[138]. Thus, the v-ErbA oncoprotein is thought to repress constitutively, through its dominant negative activity, a certain set of genes that prevent cellular transformation. Furthermore, it has been shown that transgenic male mice expressing v-ErbA had hypothyroidism with inappropriate TSH response and development of HCC [161].Further studies reported that mutated or truncated forms of TRs are expressed at high frequencies in human HCCs and in human hepatoma cell lines. These mutant receptors display a loss of transcriptional

activities, defects in the release and binding of ligand-driven co-repressors, and act as dominant-negative forms hampering the activity of the wild-type receptor [159, 162-165].However, in spite of the previously described unusual high percentage of mutations of THRα and THRβ genes, recent studies did not confirm these data. Accordingly, studies based on whole genome deep-sequencing analysis, did not detect mutations of TRs in human HCCs of different etiology [166-168] or in rat HCCs [169].

Studies conducted in hepatoma cell lines and in human HCCs have indicated a role for TH receptors in the induction of the tumor suppressor Dickkopf (DKK)4,a secreted protein, that antagonizes the Wnt signal pathway.T3/TR axis seems to play a suppressor role by inducing DKK4 expression in HCC cells at both the messenger RNA (mRNA) and protein levels [170].Moreover, further studies demonstrated the involvement of TRs in the repression of proto-oncogenes such as Sp1 and PTTG1 [170, 171].

Exceptional attention has been given to the role of TRβ1 isoform, representing the most abundant TR in hepatic tissue. When re-expressed in hepatocarcinoma cell lines with elevated Ras activation, TRβ1 showed a strong effect as suppressor of invasiveness and metastasis formation. Indeed, TRβ1-transfected human SK-hep1 cells displayed a reduced HCC xenograft tumor growth when injected in nude mice, promoted partial mesenchymal-to-epithelial transition, attenuated tumor cell invasiveness, and blocked tumor cell responses to growth factors EGF, IGF-1, and TGF-β [172]. Martinez-Iglesias *et al.*[145] reported that while growing tumors were found to lose TRβ1 expression, induction of hypothyroidism was associated with reduced tumor enlargement as well as an increased invasive and metastatic phenotype. On the other hand, a less recent study showed that expression of TRβ1 is highly correlated with increased invasiveness in human HCC cell lines and decreased expression of the anti-metastatic gene nm23 [173].

Interestingly, Frau and colleagues [169]reported a condition of local hypothyroidism in liver pre-neoplastic lesions obtained in rats subjected to the R-H protocol of hepatocarcinogenesis. The down-regulation of TRs, especially TRβ1associated to severely reduced *Dio1* expression has been shown to be an

early and significant event in liver cancer progression. Decreased expression of TRβ1 led to diminished transcription of its classical target gene *Dio1*; in turn, *Dio1* inhibition results in a reduced T4 to T3 conversion causing local hypothyroidism. Notably, down-regulation of TRβ1 was particularly evident in the most aggressive lesions, endowed with a higher proliferative capacity, further supporting the relevance of the hypothyroid status in cancer development.

In fact, the increase of TRβ1 and Dio1 levels, following T3 treatment, is associated with pre-neoplastic nodule regression, suggesting that the reactivation of the T3/TR axis may impact on the fate of pre- and neoplastic lesions **(Fig.10)**[174].

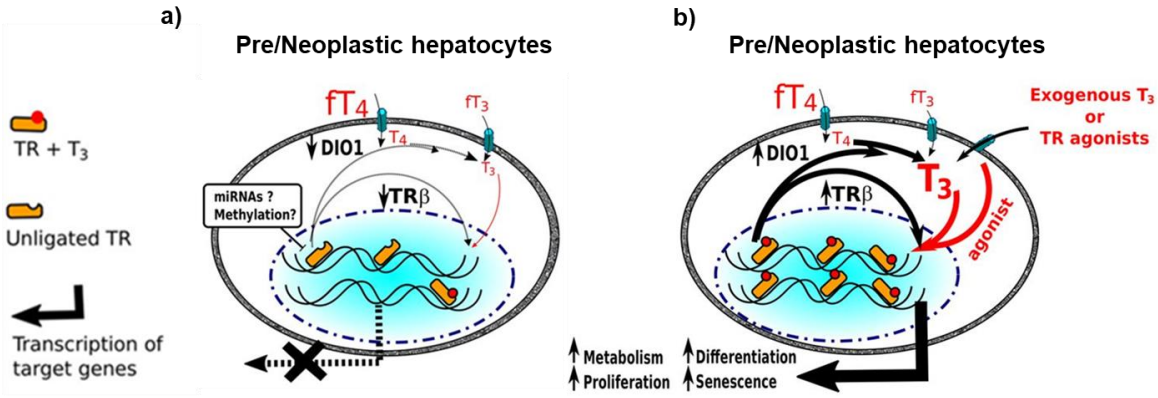

Figure 10. **a)** Hypothyroid status of preneoplastic/neoplastic hepatocytes. **b)**The reactivation of T3/TR axis following exogenous T3 or TR agonists administration and its effect on pre/neoplastic lesions.[174]

According to the potential role of local hypothyroidism in HCC development, it follows that increasing the intracellular levels of T3 might represent a novel therapeutic approach to interfere with the development of this tumor.

1.10 T3/TR axis involvement in the differentiation of pre- and neoplastic hepatocytes

As previously described, it has been shown that T3-administration stimulates the regression of hepatic preneoplastic lesions generated by the R-H protocol of hepatocarcinogenesis, showing that in addition to its mitogenic capability, T3 stimulates the remodeling/regression of nodular hepatocytes inducing a re-differentiation program of preneoplastic cells[135].

These findings are supported by the observation that while the biochemical phenotype of preneoplastic hepatocytes closely resembles that of fetal or neonatal hepatocytes, the biochemical phenotype of the nodules following T3 administration resembles that of fully differentiated normal hepatocytes. Based on these results, it was suggested that T3-induced differentiation of preneoplastic hepatocytes may be the mechanisms responsible for the anti-tumoral effect of the hormone.

In this contest, it is of interest to note that hepatocyte nodules do not express enzymes normally present in differentiated hepatocytes such as P-450, ATPase, glucose-6-phosphatase, while exhibiting high levels of enzymes expressed at a low level or absent in fully differentiated hepatocytes (γ-glutamyl transpeptidase, glutathione S-transferase P, glucose-6-phosphate dehydrogenase, α-fetoprotein)[175]. According with these findings, Enomoto and colleagues [65], showed that during the carcinogenic process, most preneoplastic lesions went through a slow regression over time and their immature phenotype was replaced by the acquisition of adult differentiated features accompanied by architectural remodeling to normal-appearing liver.

In support to these evidences, Laszlo *et al.*[176], described the ability of T3 to transiently accelerate the proliferation of the oval cells. Interestingly, proliferation was followed by a rapid differentiation of the oval cells into small hepatocytes. Positivity for hepatocyte nuclear factor-4 (Hnf4-α) and hepatocyte specific connexin 32, α1 integrin, Prox1, cytochrome P450s occurred in oval cells after the treatment. At the same time, oval cell specific OV-6 and α-fetoprotein expression was lost.

The increase of hepatocyte specific mRNAs: albumin, tyrosine aminotransferase and tryptophan 2,3-dioxygenase detected by real-time PCR also proved hepatocytic maturation.

Recent studies highlighted the potential significance of T3-induced differentiation in modulating tumor development through its interaction with Krüppel-like factors (KLFs)**.** KLFs belong to an evolutionarily conserved family of transcription factors containing a zinc finger motif [177]. Experiments *in vitro* and *in vivo* have demonstrated the importance of KLFs in proliferation, apoptosis, differentiation, cell migration, and several pathologic processes[178-180]. Indeed, KLFs create a complex network together with other family members and transcriptional activators and repressors to guarantee a correct cell behavior in several contexts. Therefore, it is not surprising that disruption of proper KLF function is involved in many pathologies. Because of their different roles in cell function, many KLFs have been implicated as key players in cancer development and progression.

In particular, KLF9 is a sequence-conserved 244 amino acid member of the KLF family whose gene is located on chromosome 9 in humans and on chromosome 19 in mice [181]. It is ubiquitously expressed and Klf9 mRNA has been identified in the bladder, brain, epidermis, gut, and heart during murine embryonic development [182]. Additionally, it has been detected in the kidneys, liver, lungs, thymus, spleen, testes, and gut of adult mice[181, 183].

KLF9 is the only member of the KLF family known to be thyroid hormone-inducible. Indeed, its expression has been reported in rat brain tissue, where KLF9 is responsive to T3 and plays an important role in T3-induced neurite growth [184]. Another study demonstrated a dose-dependent up-regulation of KLF9 by T3 in mouse brain due to the presence of a functional T3 response element (TRE) binding the TR-RXR heterodimer with high affinity [184].

KLF9 has been shown to be induced by T3 in the liver as well [185]. Interestingly Ohguchi and colleagues [186], showed that KLF9 cooperates with HNF4α and GATA4 to synergistically activate the mouse iodothyronine deiodinase I (Dio1) promoter. As previously described, Dio1 is a selenoenzyme catalyzing the bioactivation of thyroid hormone and it is highly expressed in the liver.

Cvoro *et al*.[187], showed that T3 and KLF9 target genes influenced pathways involved in stem cell self-renewal and differentiation, including Notch signaling. This pathway cross-talks with others including Wnt, FGF, TGF-β/BMP and Hedgehog [188] and converges on a transcriptional network that involves OCT4, NANOG and SOX2 to regulate stem cell maintenance and differentiation [189].

Moreover, Cvoro and colleagues [187] demonstrated that T3 works through TR to induce KLF9 in multiple cell types of hepatocyte origin and stem cells. TR activation leads to KLF9 induction in HepG2 cells, non-transformed liver cells, human induced pluripotent stem cells (hiPSC), and in human embryonic stem cells (hESCs) and this effect persists during hiPSC and hESC differentiation to definitive endoderm and mature hepatocytes. T3 action on KLF9 in ESCs occurs in the context of widespread TR-dependent effects on genes that are implicated in early stages of ESC differentiation, suggesting that the TR/KLF9 axis plays important roles during several stages of the hepatocyte lineage and in the choice between stem cell renewal and differentiation. A deeper knowledge of the T3/TR/KLF9 pro-differentiation effect would be desirable, since many studies reported the effect of KLF9 levels on HCC. Fu *et al.*[190], found a decrease in the mRNA and protein levels of KLF9 in HCC compared to surrounding normal tissue. Additionally, the same authors showed KLF9 ability to inhibit cell proliferation and mobility and to induce apoptosis in HepG2 cells through the expression of the programmed cell death protein 5 (PDCD5). In according with these findings Sun *et al.*[191], reported that a restoration of KLF9 levels significantly inhibited the growth and caused apoptosis in SK-Hep1 and HepG2 cells. Furthermore, KLF9 positively regulated p53 levels binding to GC boxes in the proximal region of the p53 promoter. Remarkably, ectopic expression of KLF9 was sufficient to delay the onset of tumors and to promote regression of the established tumors *in vivo*, suggesting that KLF9 plays a critical role in HCC development and that pharmacological or genetic activation of KLF9 may have potential in the treatment of HCC.

Taken together these findings shed light on the importance of future investigations on the T3/TR/KLF9 axis in preneoplastic and neoplastic hepatocytes to obtain more insights into the ways by which TRs modulate HCC development, possibly by activating a differentiation program.

2. AIM OF THE STUDY

A deeper knowledge of the early molecular mechanisms driving the development of HCC is strongly needed for the improvement of both diagnosis and treatment of this cancer. Previous studies performed in our laboratory showed that one-week exogenous administration of T3 in rats subjected to the R-H model of hepatocarcinogenesis and bearing hepatic preneoplastic nodules, exerts a strong antitumoral effect, inducing a rapid disappearance of the vast majority of the lesions. Consequently, the first purpose of the present study was **to identify the mechanisms induced by T3 and responsible for the regression of preneoplastic lesions.**

Moreover, since in humans the diagnosis of HCC is too often made at late stages when there is virtually no effective treatment that would improve patient's survival, a second aim of the present PhD thesis **was to investigate whether HCCs are responsive to the anti-tumoral effect of T3 as well**. To this aim, we planned another experiment to study the effect of T3 administration in rats exposed to the R-H protocol at a time when early HCCs were already developed.

3. MATERIALS AND METHODS

Animals

Male Fisher F-344 rats weighing 100-125 gr were obtained from Charles River (Milan, Italy). Animals have been fed a rodent standard diet (Standard Diet 4RF21, Mucedola, Milan, Italy) and maintained at 25°C temperature and 12 hours light/dark daily cycle, with food and water *ad libitum.* During the whole experimental period, the Guidelines for the Care and Use of Laboratory Animals were strictly followed, and all the animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health.

Experimental protocol 1 (Fig.11). Rats were injected i.p with a single dose of the carcinogen diethylnitrosamine (DENA) (Sigma Aldrich, St. Louis, MO, USA), dissolved in saline, at the dose of 150 mg/kg body weight. After a recovery period of 2 weeks, all animals were treated in accordance to the Resistant Hepatocyte protocol of hepatocarcinogenesis [62]. Rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF) (Sigma Aldrich, St. Louis, MO, USA) for 1 week and then subjected to a standard two-thirds partial hepatectomy (PH)[192] and kept for an additional week on the 2-AAF-containing diet. Five weeks after 2-AAF release, animals were randomly divided into two groups: a group was kept in basal diet (Standard Diet 4RF21, Mucedola, Milan, Italy) while the other was fed a T3-supplemented diet (4 mg/kg of T3) for 2 or 4 days.

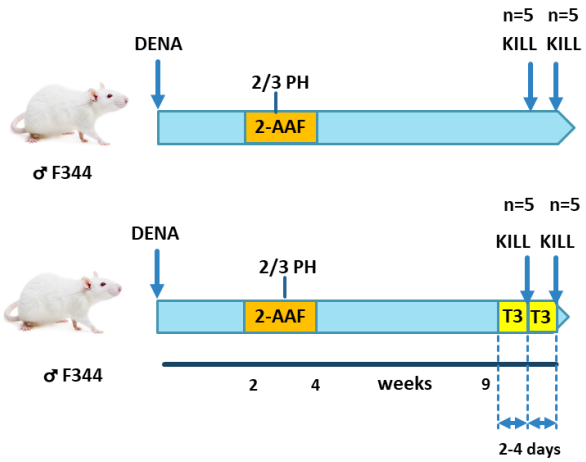

Figure 11

Experimental protocol 2 (Fig.12). Animals were subjected to R-H protocol as described in Experimental protocol 1. Following 2-AAF withdrawal, the animals were kept in basal diet for 10 months and then randomly divided into two groups: the first one was left on basal diet for another week, while the second group was exposed to a one-week treatment with T3 (4mg/kg of diet).

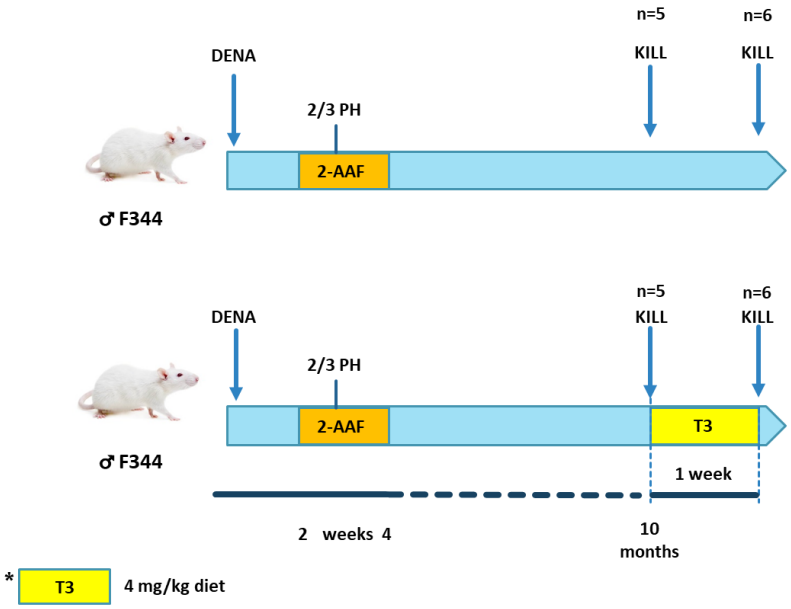

Figure 12

Experimental protocol 3 (Fig.13). Animals exposed to RH-protocol and shifted to basal diet for 10 months were randomly divided into two groups: the first group was maintained in basal diet whereas the second group was exposed to 5 cycles of T3-diet (one-week cycle/every three weeks). All animals were sacrificed 14 months after treatment with DENA.

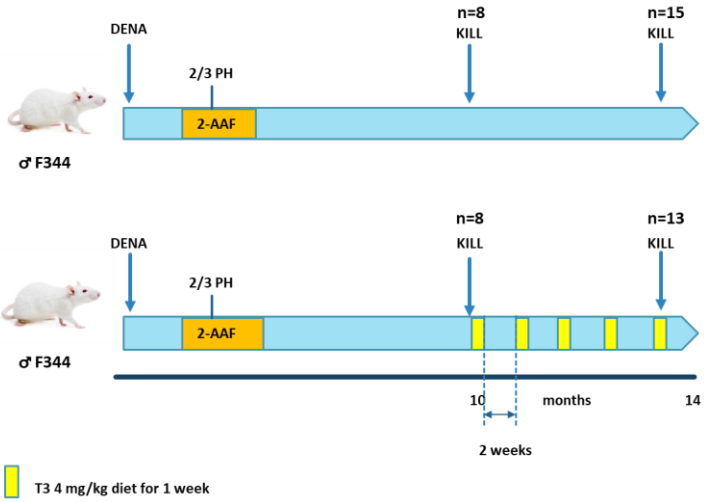

Figure 13

4. HISTOLOGY and IMMUNOHISTOCHEMISTRY

Tissue preservation

Immediately after the sacrifice, livers were cut in several pieces and preserved in different ways to carry out multiple analysis. For immunohistochemistry analysis, liver sections were fixed in 10% formalin, embedded in paraffin and stored at RT. Other sections were immediately frozen in liquid nitrogen– cooled isopentane and preserved at -80°C for future molecular analysis and cryosectioning.

Hematoxylin and Eosin (H&E) staining

Four micron-thick paraffin-embedded liver sections were deparaffinized in Bioclear (Bio-Optica, Milan, Italy) for 30 minutes and hydrated in a decreasing series of alcohol. Sections were then incubated in Carazzi Hematoxylin for 22 minutes and after several washes in tape water, stained in 1% acidified alcoholic eosin for 14 seconds. Sections were then dehydrated through ascending alcohol series, cleared with Bioclear, air-dried and then mounted using synthetic mounting and coverslipped.

Glutathione S-transferase (GST-P) staining

4 µm formalin-fixed sections were deparaffinized in Bioclear for 1 hour and hydrated in decreasing series of alcohol. Following two washes in phosphate buffered saline (PBS), the blocking of unspecific antibody binding sites was performed in 10% normal goat serum (Abcam, ab7481) for 30 minutes at room temperature (RT). The next step was the overnight incubation with 1:1000 diluted anti-GSTP antibody (MBL, 311) at 4°C in humid chamber. To block endogenous peroxidase activity, slides were incubated in 0,5% hydrogen peroxide (Sigma-Aldrich) in distilled water for 10 minutes. Sections were then incubated with anti-rabbit Horseradish Peroxidase (HRP) secondary antibody(Sigma-Aldrich) at 1:300 dilution for 40 minutes at RT. Positive binding reaction was visualized using VECTOR NovaRED Peroxidase Substrate Kit (SK-4800, Vector Laboratories) for 4 minutes at RT. Afterwards, slides were counterstained with Harris Haematoxylin Solution (HHS32, Sigma-Aldrich), dehydrated through graded alcohols, cleared and coverslips were mounted with synthetic mounting media.

Cytokeratin-19 (KRT-19) staining

Four-micrometer-thick sections were deparaffinized by a treatment with Bioclear and rehydrated in an ethanol series. For antigen retrieval, slides were microwaved in citrate buffer followed by blocking of unspecific site as previously described [193]. Endogenous peroxidases were blocked with 0,5% hydrogen peroxide (Sigma-Aldrich) in distilled water for 10 minutes. Anti-KRT-19 antibody **(**NBP1-78278, Novus Biologicals) was applied overnight at 4°C at 1:400 dilution. Sections were then incubated with anti-rabbit HRP secondary antibody at 1:300 dilution for 30 minutes at RT. Staining was revealed by using VECTOR NovaRED Peroxidase Substrate Kit (SK-4800, Vector Laboratories). Counter staining was performed with Harris Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO, USA). Finally, sections were dehydrated in ascending series of alcohol and mounted with coverslip.

Hepatocyte nuclear factor4-α (Hnf4-α) immunofluorescence staining

Six micrometer-thick (6 um) isopentane-frozen liver sections were fixed with methanol for 10 min and dried for 10 min at room temperature. Slides were then rinsed four times in in PBS-1x for 5 min each. Block specimen was performed in blocking buffer (1X PBS/ 10% donkey serum) for 30 min at room temperature. Anti-HNF4-α antibody (sc-6556, Santa Cruz Biotechnology) was applied overnight at 4°C at 1:400 dilution. Sections were washed with PBS-1x and incubated for 2 hours with the secondary antibody conjugated to Alexa FluorR 488 donkey anti-goat IgG (H+L) at 1:500 dilution (Life Technologies). Liver sections were then counter stained with DAPI for 10 minutes at room temperature (d1306, Thermo Scientific). Finally, sections were rinsed in PBS and mounted with anti-fade mounting media.

5. CYTOMETRIC ANALYSIS

At least five H&E stained liver sections for each animal were analyzed under light microscope. Neoplastic tissue was selected following the histological criteria in **Table 2**. To measure the neoplastic area we used ImageJ, a computer-assisted image processor, according to Abramoff *et al*.[193].

Table 2. Histological Criteria defining Neoplastic Areas

Lesion size
Invasion of stroma or portal tracts
Nuclear-cytoplasmic ratio
Nuclear atypia
Cytoplasmic modifications (size, basophilia, texture, glycogen/lipid accumulation)

6. LASER CAPTURE MICRODISSECTION

Sixteen-µm-thick serial frozen sections were cut and attached to 2-µm RNase-free PEN-membrane slides (Leica, Bannockburn, IL). To identify the localization of pre-neoplastic lesions, six µm thick sections were cut and stained for H&E, GST-P and KRT-19 to recognize positive lesions. Immediately before performing micro-dissection, frozen sections were rapidly stained with a 3½ minutes H&E procedure. Briefly, sections were hydrated (30 seconds in Ethanol 100 and 95%), stained in Mayer's hematoxylin for 90 seconds, washed in water for 20 seconds, stained in 0.25% alcoholic Eosin for 10 seconds and dehydrated by Ethanol 100% for 30 seconds. Micro-dissection was performed using a Leica laser microdissection apparatus (LMD6000) **(Fig14)**. The whole procedure was performed within 20 minutes to avoid RNA degradation. To guarantee the maximus amount of material, the same lesion was identified and cut from 5 to 10 serial sections. Dissected material from the same nodule was collected in the same 0.5 ml micro centrifuge tube's cup filled with 50 µl of Lysis/Binding Buffer. At the end of the procedure, microtubes were plugged up, span to collect dissected material and immediately frozen at -80°C until extraction with mirVana™ miRNA Isolation Kit (Ambion, AM1560).

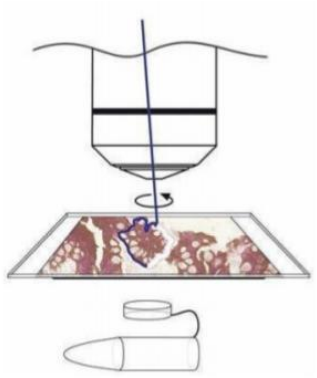

Figure 14. Microdissection with Leica laser microdissection apparatus (LMD6000)

7. RNA EXTRACTION

RNA extraction from preneoplastic lesions

Total RNA was extracted from preneoplastic lesions and from corresponding control livers with mirVana miRNA Isolation kit (mirVana, Ambion, Life Technologies, Monza) according to manufacturer‘s instructions. Briefly, micro-dissected lesions were dissolved in 300 µL of Lysis Buffer (LB) and 30 µL of miRNA Homogenate Additive. After a 10 minutes incubation on ice, organic extraction of RNA was performed adding 300 µL of acid-phenol:chloroform:isoamyl alcohol (125:24:1) to each sample. Samples were then centrifuged for 5 minutes at maximum speed to separate the aqueous and organic phases. After recovery of the RNA-containing aqueous phase, 1.25 volumes of 100% ethanol were added, and the mixture was transferred to a Filter Cartridge. Collecting tubes were centrifuged and the flow-through was discarded. Following three washing steps with Wash Buffers, filters were dried by a 60 seconds full speed centrifugation. Finally, RNA was eluted with pre-heated (95°C) RNAse-Free distilled water and stored at -80°C.

RNA extraction from rat HCCs and control livers

Sixteen-µm-thick frozen sections were cut from different pieces of each animal liver to represent in the best way the whole sample. All the sections were then collected in 0.5 ml tubes and stored at -80°C until extraction. Total RNA from ratadvanced HCCs (14 months after DENA) and control livers was isolated using the mirVana miRNA Isolation kit as previously described.

8. QUANTITATIVE AND QUALITY ANALYSIS OF NUCLEIC ACIDS

Total RNA concentrations and purity ratios (260/280 and 260/230) were measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, France). RNA integrity was evaluated by Agilent Bioanalyzer 2100 (Agilent Technologies) by assessing the RNA Integrity Number (RIN). Only RNA samples with a RIN equal to or higher than 7 were further used in the study. All procedures were performed according to manufacturer’s protocol.

9. MICROARRAY

Gene expression profile analysis was performed using the RatRef -12 V1 Beadchip Illumina, containing 21791 gene-specific oligonucleotides. Four Chips were loaded to investigate 48 samples divided in the following groups:

- 8 samples were obtained by the micro-dissection of untreated rat liver tissue as matched- age absolute controls.
- 10 samples consisted in a pool of KRT-19 positive pre-neoplastic lesions micro-dissected from rats subjected to R-H protocol and sacrificed respectively at 2 and 4 days after 9weeks DENA initiation;
- 10 samples were obtained by micro-dissecting a pool of KRT-19 positive pre-neoplastic lesions from rats subjected to R-H protocol and fed a T3 diet for 2 days;
- 10 samples were obtained by micro-dissecting a pool of KRT-19 positive pre-neoplastic lesions from rats subjected to R-H protocol and fed a T3 diet for 4 days
- 10 samples were collected by micro-dissecting a pool of KRT-19 negative pre-neoplastic lesions from rats subjected to R-H protocol.

RNA Amplification

Amplification of 150 ng of total RNA was performed with an Illumina® TotalPrep RNA Amplification Kit (Ambion, Life Technologies, Milano) The Illumina® TotalPrep RNA Amplification allows to generate biotinylated, amplified RNA for hybridization with Illumina Sentrix® arrays. The protocol consists of a reverse transcription with an oligo primer bearing a T7 promoter using a reverse transcriptase to produce higher yields of first single-strand cDNA. Afterwards, cDNA goes through a second strand synthesis and it is cleanup to become a template for in vitro transcription (IVT) with T7 RNA Polymerase. During this step biotin-UTPs are used to generate a great amount of biotinylated, antisense RNA copies (cRNA). The labeled cRNA produced with this procedure are then use for the hybridization with Illumina arrays.

BeadChips Illumina hybridization

A total of 750 ng biotinylated cRNA were hybridized for 18 hrs to RatRef-12 V1 BeadChip. Hybridized chips were washed and stained with streptavidin-conjugated Cy3 (GE Healthcare Milano, Italy). BeadChips were dried and

scanned with an Illumina BeadArray Reader (Illumina Inc., San Diego, CA, USA).

Microarrays data analysis

For data analysis, the intensity files were loaded first into the BeadStudio 3.0.14 Software (Illumina Inc., San Diego, CA, USA) to extract the fluorescence values and for quality control analysis. The extracted values were then loaded BRB Array Tools (Version 4.4.0) for gene expression analysis. First, the quantile normalization algorithm was applied on the dataset to correct systematic errors. Using a normalization based upon quantiles, this method normalizes a matrix of probe level intensities.

Only genes whose expression differed by at least 1.5-fold from the median in at least 20% of the arrays and characterized by a 50th percentile of intensities greater than 300 were considered as detected. According to these criteria, 869 expressed transcripts out of 21.791 showed reproducible up- or down-regulation. Hierarchical cluster analysis (Heat-Map) divided the experimental groups in different clusters depending on their grade of expression profile similarity. Data were organized as a tree diagram called dendrogram in which the distance between the branches represented the dissimilarity among the groups. In this map, green color indicates a low fluorescence intensity, representing downregulated elements whereas red color indicates a high fluorescence intensity representing upregulated elements.

F-test (with random variance model) and multivariate permutation test (Confidence level of false discovery rate assessment: 80 %, Maximum allowed proportion of false-positive genes: 0.1) were used to identify genes differentially expressed. Following this analysis, 864 genes were differently expressed.

IPA (Ingenuity Pathway Analysis)

Genes were classified according to their role in biological process, cellular components, and molecular function using Ingenuity Pathway Analysis (IPA), a web-based functional analysis tool developed by Ingenuity Sistems (www.ingenuity.com). IPA is a powerful resource to identify the most relevant signaling pathways, molecular networks and biological functions for genes of interest. Moreover, it can predict the activation and inhibition of upstream

transcription factors and the direction of downstream effects on biological and disease processes. Pathway analysis was carried out on genes found to be significantly modified (cut off up-regulated>+2 e down-regulated < -2). Afterwards, genes were correlated to specific biological functions and it was possible to identify transcription factors implicated in the expression of the genes present in our dataset.

10. ANALYSIS OF mRNA EXPRESSION LEVELS

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To investigate by quantitative real time-PCR (qRT-PCR) mRNA expression levels of *HNF4α*, *KRT-19, Dio1, G6pc, Klf9 and Cebpα,* total RNA was retro-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Life Technologies, Italy).

The reaction mixture contained: 2 µl of RT buffer (10X), 2 µl of Random Primers (10X), 0.8 µl of dNTP mix (100 mM), 1 µl of MultiScribe Reverse Transcriptase, 1 µl of RNase Inhibitor and 3.2 µl of DNase/RNase-free distilled water and 10 µl of the appropriate total RNA at the desired concentration. Thermo cycle condition was: 25° C for 10 minutes, 37° C for 120 minutes and 85° C for 5 minutes, followed by a 4° C hold. Samples were then stored at -20° C until next use.

11. QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Retro-transcribed cDNAs were used for the assessment of mRNA gene expression analysis performed by qRT-PCR. The amplification reaction was performed in a final volume of 10 µl mixture containing: 4 µl of cDNA template (2 ng/µl), 5 µl of 2X TaqMan Gene Expression Master Mix (Applied Biosystem, Life Technologies, Italy), 0.5 µl of 20X TaqMan assay (Applied Biosystem, Life Technologies, Italy) and 0.5 µl of RNase-free water.

The following TaqMan probes were used:

-Rn4339144_m1 for the analysis of rat *HNF4a* (Hepatocyte nuclear factor a);

-Rn01496867_m1 for the analysis of rat *KRT-19* (Cytokeratin-19);

-Rn00572183_m1 for the analysis of rat *DIO1* (Deiodinase 1);

-Rn00689876_m1 for the analysis of rat G6PC (Glucose-6-phosphatase);

-Rn00589498_m1 for the analysis of rat *KLF9* (Kruppel like factor 9);

-Rn00560963_s1 for the analysis of rat*CEBPa*(CCAAT/enhancer binding protein alpha)

Reactions were performed in a ABI PRISM 7300HT thermocycler (Applied Biosystem, LifeTechnologies, Italy); the cycle conditions were set as follows: 50° C for 2 minutes and 95° C for 10 minutes, followed by 40 cycles at 95° C for 15 seconds and at 60° C for 1 minute. Each sample was analyzed in triplicate; the housekeeping gene rat GAPDH (glyceraldehyde-3 phosphate dehydrogenase) was used for normalization. To determine the relative expression levels of *HNF4α*, *KRT-19, Dio1, G6pc, Klf9 and Cebpα* the 2−ΔΔCT method was used. The threshold cycle (Ct) value of the target gene was normalized to that of the endogenous reference and compared with a calibrator.

12. STATISTICS

Data are expressed as mean ± standard error (SEM). Analysis of significance was done by t Student‘s test and by One-Way ANOVA using the GraphPad software (La Jolla, California).

13. RESULTS

T3 administration induces a switch of the genetic profile of KRT-19+ preneoplastic lesions towards that of fully differentiated hepatocytes

Previous studies from our laboratory have shown that while only 25-30% of GST-P+ preneoplastic nodules are positive for the putative progenitor/stem cell marker KRT-19, almost all HCCs show positivity to this marker, suggesting their origin from KRT-19+ preneoplastic nodules [66]. Support to this hypothesis stems from the similar expression profile observed between KRT-19+ nodules and HCCs [67]. Thus, we investigated the effect of T3 on KRT-19 positive lesions, performing a transcriptomic analysis of laser-microdissected preneoplastic nodules at 2 and 4 days after T3 treatment. Gene expression profiling, performed using the Illumina platform, included a total of 869 differentially expressed genes out of 21.791 present in the array. As shown by the heat map **(Fig.15)**, unsupervised hierarchical analysis grouped rat lesions in two principal clusters. Notably, while the first one consisted of preneoplastic KRT-19+ lesions of untreated animals and of rats treated with T3 for 2 days, the second one included preneoplastic KRT-19+ lesions of rats treated with T3 for 4 days, KRT-19 negative lesions and age-matched normal controls. Thus, T3 treatment for four days triggered a striking shift of the expression profile of KRT-19+ lesions towards that of normal liver or of the well differentiated and slow-growing KRT-19-negative lesions. These results demonstrate that T3 administration induces a reprogramming of undifferentiated hepatocytes to a more mature phenotype and suggest that this could be the mechanism responsible for the rapid regression of the KRT-19 positive nodules observed one week after T3 administration.

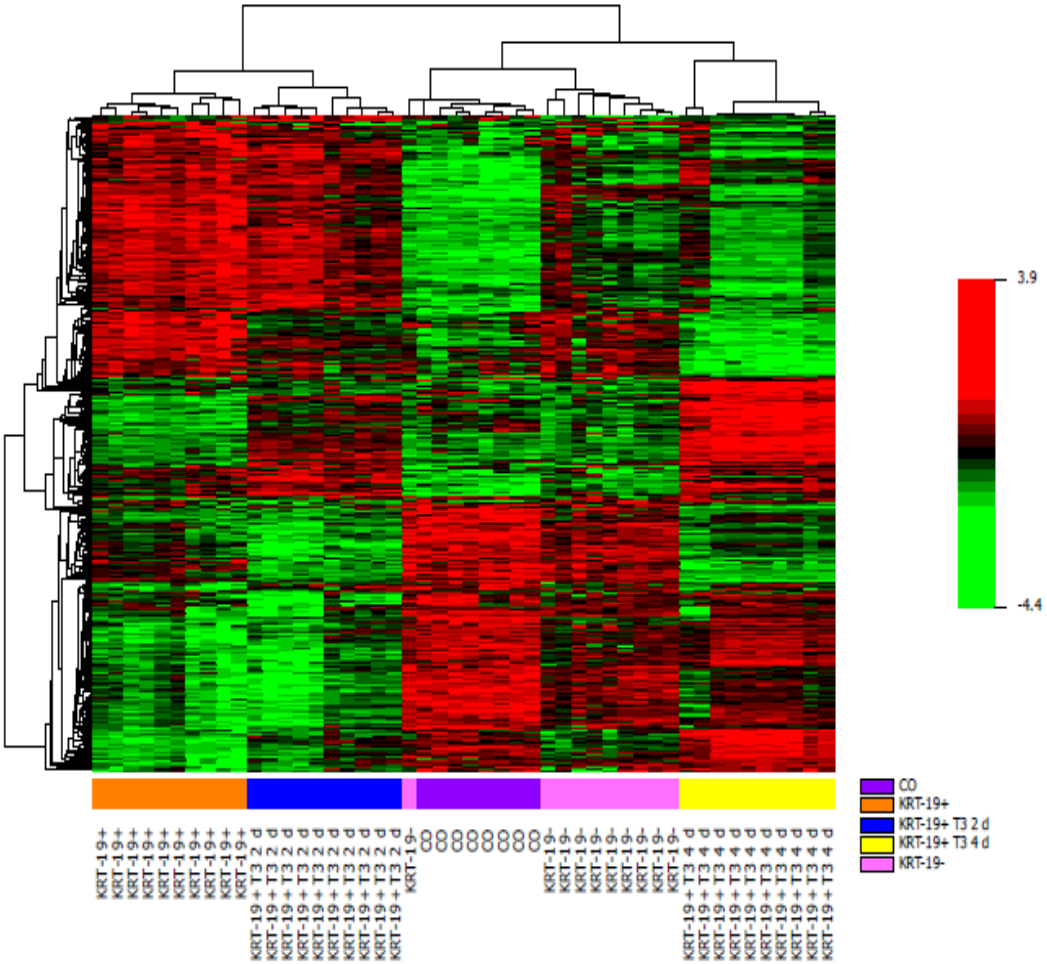

Figure 15. Unsupervised hierarchical clustering of gene expression profile in KRT-19+ nodules after 2 days T3-treatment (KRT-19+ T3 2d), age-matched controls (CO), KRT-19- and KRT-19+ nodules after 4 days T3-treatment (KRT-19+ T3 4d). Red and green colors represent higher or lower expression levels of the genes, respectively.

Activation of T3-induced transcription factors involved in hepatocyte differentiation

It has been already demonstrated that triiodothyronine works through TRs to induce and regulate transcription factors involved in hepatocyte differentiation [187]. To investigate whether the global change in the expression profile of KRT-19+ lesions could be due to a T3-induced differentiation program, we analyzed all genes differentially expressed in the array with the Ingenuity Pathway Analysis software (IPA). Strikingly, IPA suggested that T3 induced liver-enriched transcription factors such as HNF1-α and HNF4-α, responsible for modulating specific transcription networks and signaling pathways involved in the maintenance of differentiated hepatocyte phenotype[194]. Thenalyzed dataset showed that T3 administration triggered a switching-OFF/ON mechanism. In fact, while in the absence of T3 treatment HNF1-α and HNF4-α are predicted to be inhibited, the same transcription factors were activated following T3 administration **(Fig.16)**.

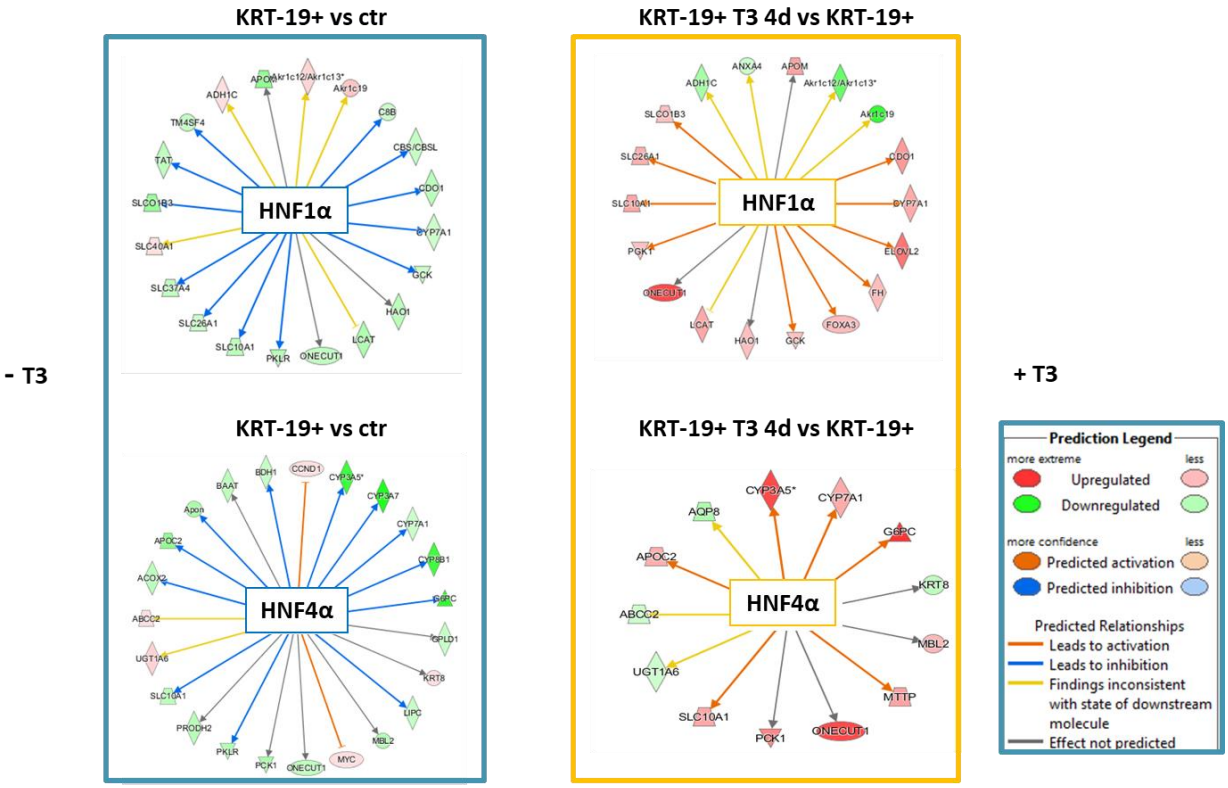

Figure 16. Network visualization of the transcription factors HNF1α and HNF4α pathway. Data and visualization were obtained using IPA. Red: up-regulated genes; green: down-regulated genes. *TF → target gene*

Analysis of mRNA levels of genes involved in hepatic differentiation

To validate the potential capability of T3 to induce hepatocyte differentiation, as suggested by IPA analysis, we determined the mRNA expression levels of *Krt-19*, a marker of de-differentiated status whose expression is associated with hepatic progenitor cells (HPCs) and early hepatoblasts [195].The expression of Klf9 and Hnf4-α, well known markers of fully differentiated hepatocytes, was also investigated. As shown in **Fig.17,** T3 treatment induced a significant down-regulation of *Krt-19* gene expression and, concomitantly, up-regulation of *Hnf4-α* and *Klf9* levels, as compared to KRT-19+ nodules of rats not exposed to T3 treatment. These changes were associated to a strong up-regulation of *Dio1* mRNA, a target of TRβ, suggesting that T3 administration restores the T3/TR axis in preneoplastic lesions, leading to the induction of genes specifically involved in hepatocyte differentiation[187].As previously reported by Frau and colleagues[169], downregulation of *Dio1* was observed in KRT-19 + preneoplastic lesions, confirming a condition of local hepatic hypothyroidism [196]. Inhibition of Dio1 levels was fully rescued by T3 administration.

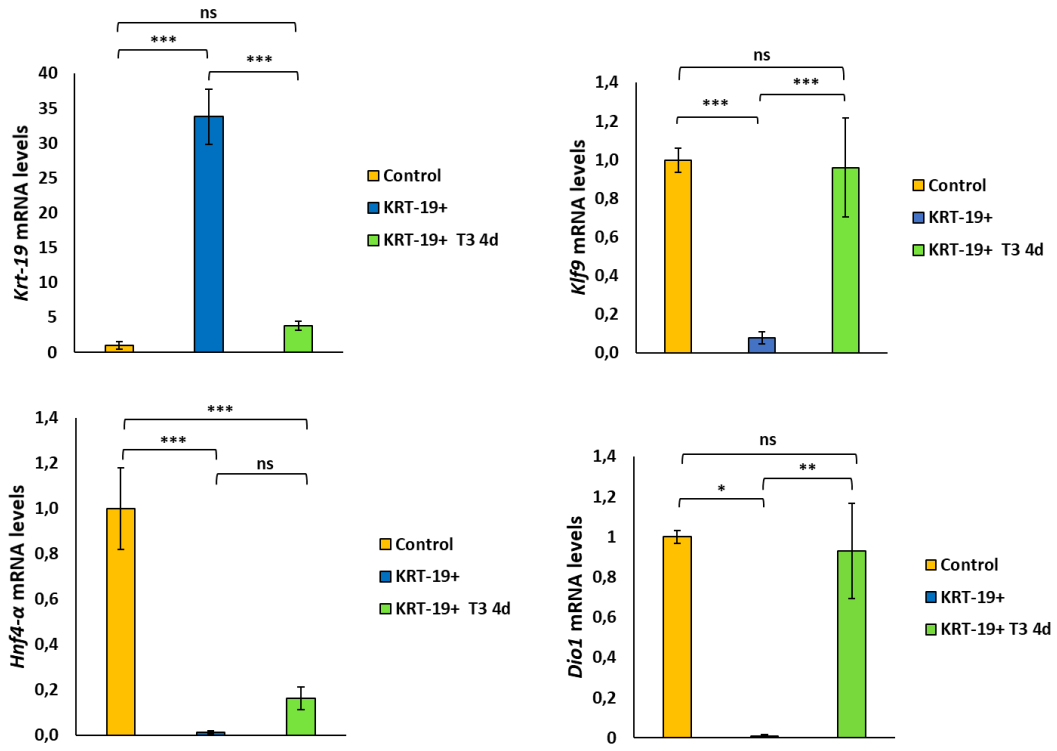

Figure 17. mRNA expression of *Krt-19*, *Klf9, Hnf4-α and Dio1* assessed by qRT-PCR. Gene levels were calculated as fold change and normalized using rat *Gapdh* as endogenous control. *** P < 0.001; ** P<0.01; * P <0.05. ns: not significant

eHCCs maintain the ability to respond to T3

In humans the diagnosis of HCC is often made at late stages when there is virtually no effective treatment.

To determine whether T3 treatment could exert an anti-tumoral effect also at late stages of the tumorigenic process, we initially investigated whether early HCCs are still responsive to T3. To this aim, animals exposed to the R-H protocol were sacrificed 10 months after DENA initiation. At sacrifice, macroscopically evident liver tumors were observed in all the animals **(Fig.18)**. From a pathological point of view these tumors showed marked cellular and mild nuclear atypia. Neoplastic hepatocytes were arranged in thick plates (up to 7 layers) or in a solid, hypercellular pattern. Moreover, a pseudoglandular pattern has been also observed. Portal tracts and central veins were occasionally seen within the lesion. Taken together, these features match with the presence of high grade dysplastic nodules or early HCCs.

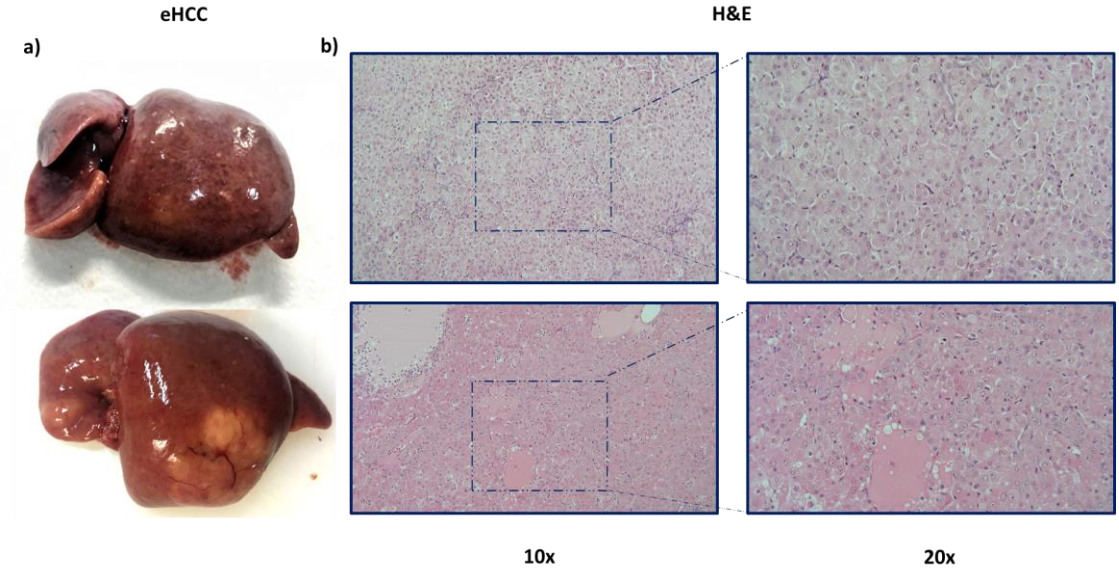

Figure 18. a) Representative gross liver images of eHCCs developed 10 months after treatment with DENA. **b)** Histological observation of the tumors by hematoxylin and eosin (H&E) staining under light-field microscope with 10x and 20xmagnification.

Gene expression analysis showed down-regulation of *Dio1* mRNA levels in eHCCs **(Fig.19a)**, demonstrating that dysregulation of the T3/TR axis affects also late stages of the tumorigenic process. A strong increase of *Krt-19* mRNA levels and of KRT-19 and GST-P protein content was also observed in the same

eHCCs **(Fig.19a, b)**. Upon treatment with T3 for 1 week, mRNA and protein levels of KRT-19 were strikingly down-regulated, as shown by qRT-PCR and immunohistochemical analyses **(Fig. 19a, b)**. Decrease of KRT-19 and GST-P was associated to a strong up-regulation of *Dio1* mRNA levels. These evidences demonstrated that T3 maintains its ability to restore the T3/TR axis and to exert its pro-differentiating effects also at late stages of the tumorigenic process.

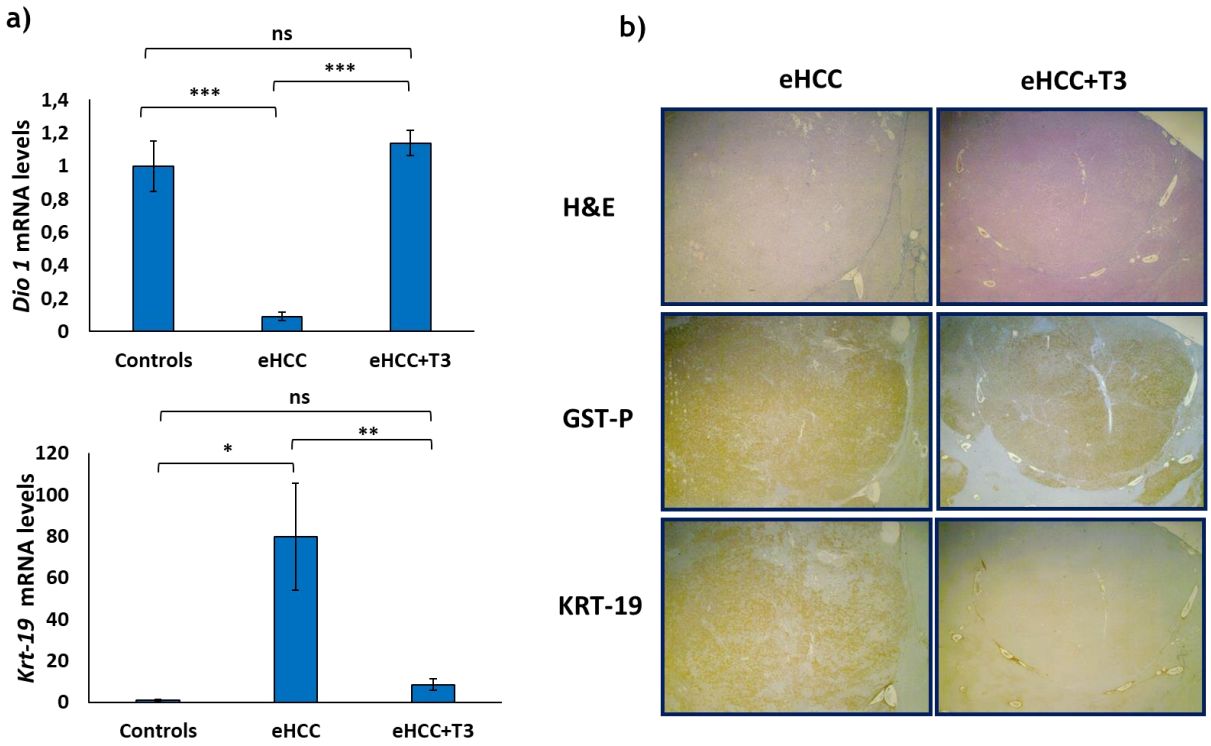

Figure 19. a) mRNA expression of *Dio1* and *Krt-19* was assessed by qRT-PCR in controls (n=3), eHCCs (n=5) and eHCCs+T3 (n=6).Gene levels were calculated as fold change and normalized using rat *Gapdh* as endogenous control. *** P < 0.001; * P <0.05. ns: not significant. **b)** Representative pictures of liver sections stained for H&E, GST-P and KRT-19 in eHCC with or without T3 administration for 1 week.

Repeated cycles of T3 induces HCC regression

Having shown that eHCCs are still responsive to T3 administration, next we addressed the question of whether T3 treatment of eHCC-bearing animals could interfere with tumor progression. To this aim, 10 months after DENA, rats were exposed to 5 cycles of T3-supplemented diet (1 week/every 3 weeks), and sacrificed 3.5 months afterwards. At sacrifice, all untreated animals exposed to the RH protocol developed multiple macroscopically evident HCCs **(Fig.20).** On the other hand, only 38,5% of T3-treated rats displayed macroscopically evident

tumors, and only 15% bore multiple HCCs **(Fig 21a)**. The significant decrease in tumor burden was associated to a reduction in liver weight/body weight ratio **(Fig.21b)**.

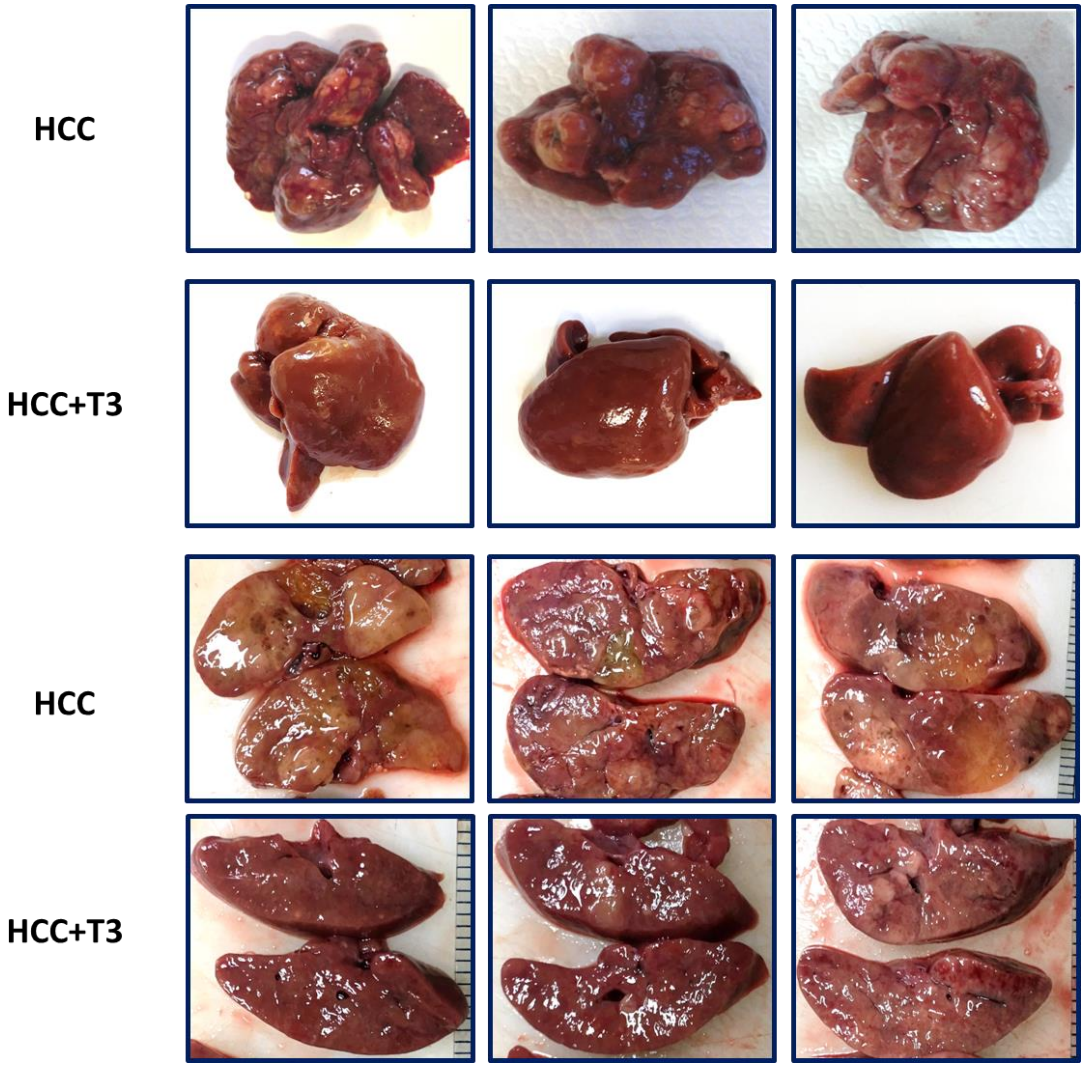

Figure 20. Representative gross liver images of rats fed T3 vs. animals fed basal diet. T3 group displays a lower number and burden of tumors.

a)

	number of rats	% rats with HCC	% rats with multiple HCCs
-T3	15	100	100
+T3	13	38,5	15

b)

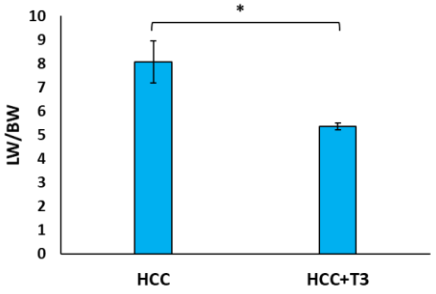

Figure 21. a) Percentage of rats showing macroscopically evident and multiple HCCs in T3- and basal diet fed rats. **b)** Decreased liver weight/body ratio (LW/BW) in T3-fed rats as compared to that of rats fed basal diet. * significantly different for P=0.02

Light microscope examination confirmed the presence of tumors, ranging from well to poorly differentiated HCCs. Neoplastic hepatocytes were arranged in thick plates (more than 5 hepatocytes thick), or showed a pseudoglandular pattern (as shown in **Fig. 22a**); on the opposite, only well differentiated and smaller HCCs were observed in the liver of rats exposed to repeated cycles of T3, strongly suggesting that thyroid hormone is able to induce a phenotypic regression of advanced neoplastic hepatic lesions **(Fig.22b)**.

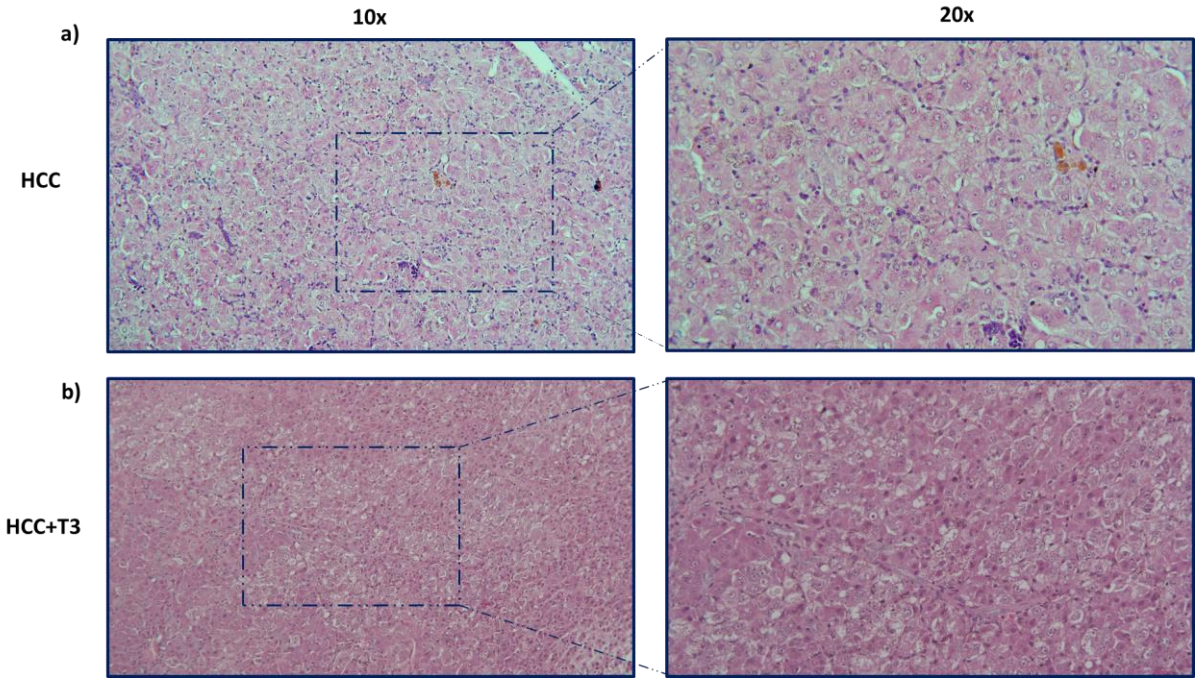

Figure 22. Representative H&E stained liver sections showing microscopic appearance of liver tumors induced by RH protocol 13.5 months after initiation in rats untreated and treated with T3.

Quantification of neoplastic areas further supports the anti-tumoral effect of T3

Next, we used the processor ImageJ to measure the neoplastic areas present in the liver of rats treated or not with T3. At least five liver sections from each animal were measured. Neoplastic tissue was selected according to the criteria described in **Table 1**. Cytometric analysis showed a significant decrease in tumor area of T3-treated samples compared to those of animals fed basal diet. In fact, while 41% of the investigate liver area was occupied by neoplastic lesions in T3-untreated animals, a strong reduction was observed in the liver of rats treated with thyroid hormone, with only 13% of the section being occupied by neoplastic lesions **(Fig.23)**.

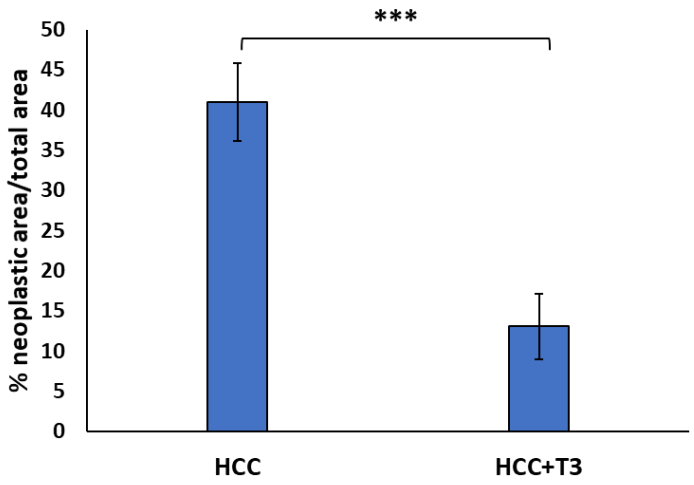

Figure 23. Graph showing the comparison between the neoplastic area/total area in liver sections of rats sacrificed 13.5 months after DENA, with or without repeated administrations of T3. Mean ± SEM**.**

T3 treatment re-establishes T3/TR axis in neoplastic hepatocytes

To establish whether the antitumoral effect of T3 on advanced neoplastic lesions could be associated with the reestablishment of the T3/TR axis, we analyzed the mRNA levels of two specific TRβ-target genes, namely *Dio1* and *G6pc* (Glucose-6-phosphatase). QRT-PCR analysis showed a strong upregulation of the expression of both the genes following T3 administration, confirming that T3 maintains its ability to restore T3/TR axis even at late stages of HCC development **(Fig.24)**.

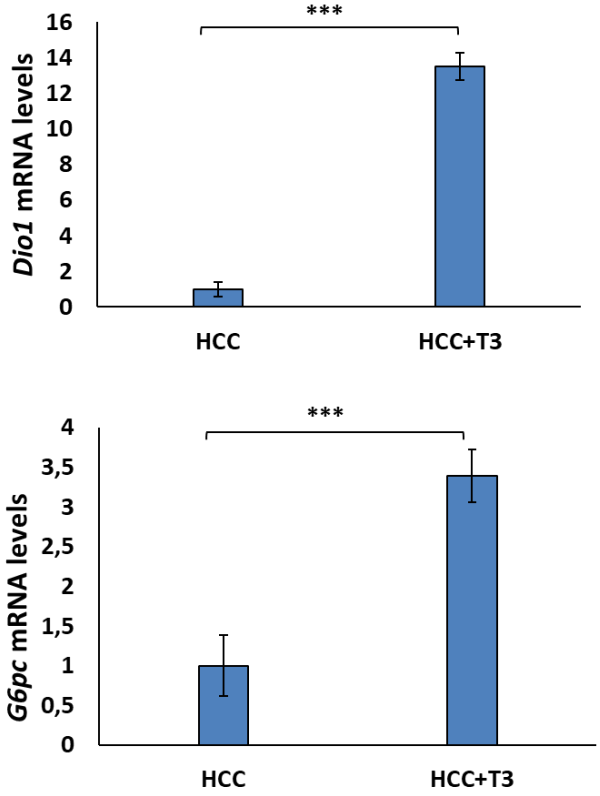

Figure 24. mRNA expression of *Dio1* and *Krt-19* was assessed by qRT-PCR. Gene levels were calculated as fold change and normalized using rat *Gapdh* as endogenous control.*** P < 0.001;

In order to investigate whether reactivation of the T3/TR axis could be associated to induction of a differentiation program, also in such advanced neoplastic lesions, we analyzed by qRT-PCR, immunohistochemistry and immunofluorescence the expression of genes involved in maintenance/loss of the differentiated status of hepatocytes.

The results showed that T3 exerted a strong stimulatory effect on the expression of transcription factors associated to hepatocyte differentiation, such as *Klf9*, *Cebpα, and Hnf4-α* **(Fig.25a, b)**, while concomitantly causing a reduction of neoplastic markers associated to less differentiated hepatocytes (GST-P and KRT-19) **(Fig. 25c**).

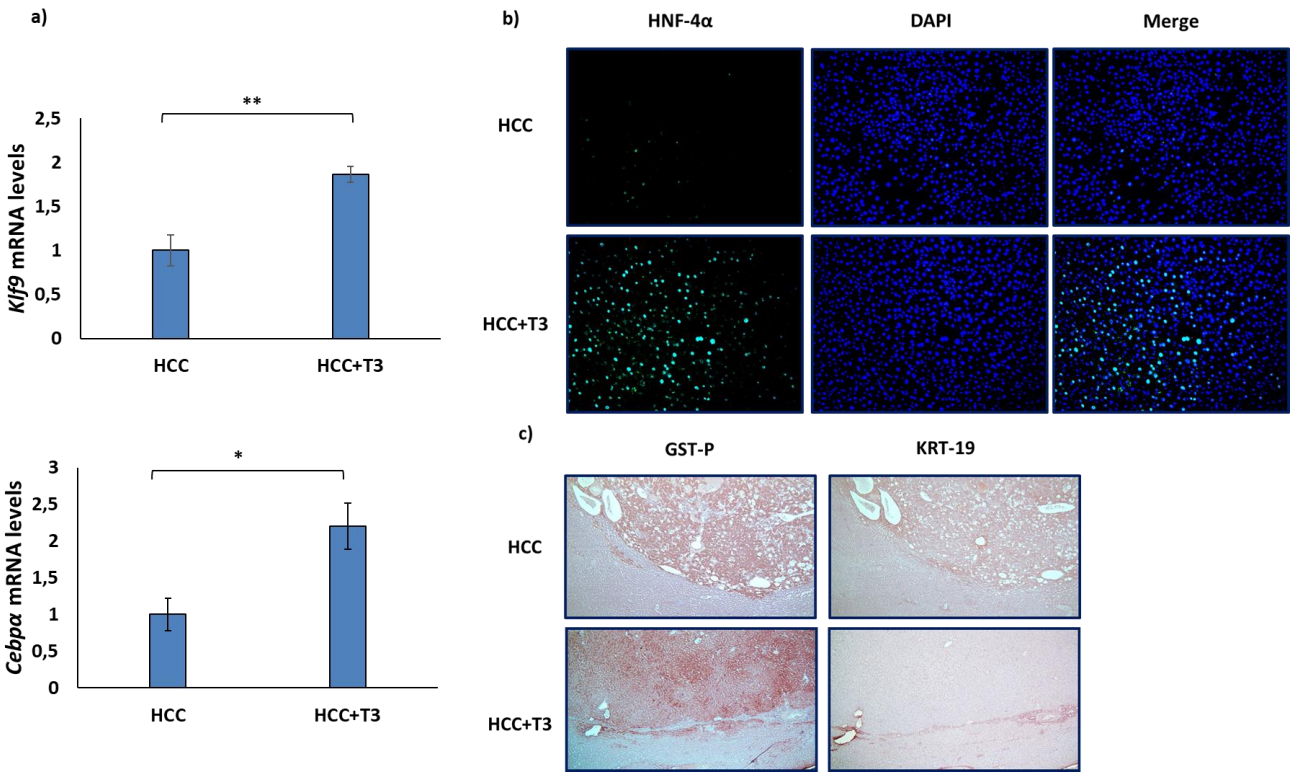

Figure 25. a) QRT-PCR analysis of *Klf9* and *Cebpα* mRNA levels. Gene levels were calculated as fold change and normalized using rat *Gapdh* as endogenous control. ** P<0.01; * P <0.05**;** **b)** Immunofluorescence staining for Hnf4a in HCCs with or without T3(20X).**c)** Representative pictures of immunohistochemical staining for GST-P and KRT-19 in HCCs with and without T3.

14. DISCUSSION

A considerable set of evidence has demonstrated that thyroid hormones plays a crucial role in carcinogenesis. In the last decades, numerous studies reported that changes in thyroid hormone levels seem to be related to an alteration in thyroid hormone receptors and/or deiodinase expression that contribute to the development of a carcinogenic milieu [174, 197]

Recent studies have shown that a partial loss of normal TRs function caused by mutation and/or aberrant expression, provides an advantageous environment for tumors to proliferate, invade other tissues and metastasize[170]. However, it remains unclear whether changes in thyroid hormone levels affect cancer development and progression. Indeed, contrasting data are reported in literature.

Population-based case-control studies of risk factors associated to development of ovarian and pancreatic cancers found that hyperthyroidism is associated with a two-fold increase of cancer risk [141, 142]. Moreover, while hyperthyroidism is associated with more advanced clinical stage and higher risk of recurrence in prostate cancer [198], a lower risk of carcinoma and a reduced progression to more invasive stages were observed for mammary cancer [146]. Consistent with hypothyroidism being beneficial, pharmacologically-induced hypothyroidism together with tamoxifen treatment, resulted in better survival of glioblastoma patients, and a significantly longer survival was observed in patients with recurrent high-grade gliomas treated with tamoxifen and the anti-thyroid drug propylthiouracil [147].

An opposite conclusion stems from two case-control studies suggesting that hypothyroidism represents a risk factor for HCC development. In one of these studies, women with a history of hypothyroidism had a 2.8-fold higher risk of HCC [158]; in the second study, hypothyroidism was significantly more prevalent in patients with HCC of unknown etiology than in HCC patients with alcoholic liver disease or HCV. Thus, these results suggest that hypothyroidism may be a permissive factor for the development of HCC[148].

A hypothyroid status of HCC has been described in human HCC [169, 170, 172]. Down-regulation of TRs, in particular THRβ1, appears to be associated with

different tumor onset and progression, including human and rat HCC. Moreover, animal studies reported that downregulation of TRβ1 is associated to a dramatic reduction of *Dio1* expression, and represents a very early event in the multistage process of hepatocarcinogenesis preceding neoplastic transformation [169]. These findings suggest that local hypothyroidism may represent a predisposing condition for the development of HCC.

On this basis, the present study was aimed at investigating whether restoration of T3/TRs axis by exogenous administration of T3 might interfere with HCC progression. To this purpose, we employed the RH protocol of hepatocarcinogenesis which allows to dissect the several steps of HCC development. By using this experimental model, we investigated: i) the possible mechanism(s) responsible for the regression of preneoplastic nodules following T3 administration, and ii) whether exogenous T3 could interfere with HCC progression also when treatment begins at a time when HCC is already developed.

As to the first question, unsupervised hierarchical analysis revealed that T3 treatment is able to trigger a striking shift in the global expression profile of KRT-19+ lesions towards that of normal liver or of the well differentiated and slow-growing KRT-19 negative nodules. Accordingly, T3 stimulation of genes responsible for the maintenance of the differentiated status of hepatocytes, such as *Hnf4-α* and *Klf9,* and inhibition of genes coding for proteins expressed in undifferentiated hepatocytes, such as *Krt-19*, was observed following treatment with T3. These modifications in the expression profile preceded the loss of the vast majority of preneoplastic nodules observed after 7 days of T3 treatment [135]. All these changes were preceded by increased expression of TRβ target genes, such as *Dio1* and *G6pc*, as a consequence of reactivation of the T3/TR axis.

These findings are in agreement with previous studies from our laboratory in which we showed that treatment of preneoplastic hepatocytes with T3 or the thyromimetic GC-1[199], led to re-expression of two enzymes, glucose 6-phosphatase and adenosine triphosphatase, expressed in differentiated hepatocytes and lost in preneoplastic lesions.

Thus, the antitumoral effect exerted by T3 on preneoplastic lesions appears to be due to the activation of a differentiation program following restoration of the T3/TR axis.

Although the T3-induced regression of preneoplastic lesions points to an anti-tumoral effect of the hormone, it is not known whether the same effect can be exerted also at late stages. This is a critical question since, in humans, no effective therapy for HCC is available when the tumor is diagnosed at late stages. Therefore, a second question we addressed was whether fully developed HCCs were still responsive to T3 effect. The re-establishment of *Dio1* mRNA levels to control values confirmed the capability of one-week T3 treatment to restore the T3/TR axis in advanced lesions. Frau *et al*.[169], showed that rat and human HCCs displayed a downregulation of TRβ1 receptor that resulted in the inhibition of the target gene *Dio1*, leading to the reduction of the rate of T4 to T3 conversion and to a negative loop responsible for the establishment of a local hypothyroidism condition. Notably, downregulation of TRβ1 was particularly evident in the most aggressive lesions, characterized by a higher proliferative capacity, further supporting the relevance of the hypothyroid status in cancer development.

Our findings revealed that T3 possesses a strong antitumoral activity also when given to animals bearing fully developed HCCs. Indeed, tumors responded to the pro-differentiation effect of this hormone, as shown by a decrease of the neoplastic markers GST-P and KRT-19 and a concomitant increase of Hnf4-α, a marker of fully differentiated hepatocytes. These findings were further reinforced by the observation that mRNA levels of genes involved in the maintenance of the differentiated status, such as *Cebpα* and *Klf9* were up-regulated following T3 administration.

Our data reporting the pro-differentiating role of T3 are in agreement with those of Cvoro and colleagues [200] showing a T3/KLF9 network as part of the re-activation of a differentiation program. In their work, the authors showed that TRs cooperate with *Klf9* to regulate hepatocyte differentiation and that TRs activation leads to *Klf9* induction in HepG2 cells, non-transformed liver cells, human induced pluripotent stem cells (hiPSC), and in human embryonic stem

cells (hESCs). Further support to the role of Klf9 stems from the finding that its downregulation has been reported in human HCCs compared with the normal liver counterpart. Remarkably, exogenous expression of Klf9 inhibited proliferation of HCC cell lines and their tumorigenic potential when xenografted in immune-depressed mice [191].

Notably, T3-induced differentiation of neoplastic hepatocytes was also evident from histological observation of the tumors of rats with or without T3 treatment. In fact, while we observed the presence of well- or poorly-differentiated HCCs in rats not exposed to T3, only well differentiated and much smaller HCCs were present in the liver of T3-fed rats. Furthermore, cytometric analysis revealed that only 13% of the total liver area was occupied by neoplastic lesions in T3-treated rats, compared with 41% of the untreated ones, indicating a complete re-differentiation towards a normal hepatocytic phenotype.

In conclusion, our data show that T3 may act as a potent anti-tumoral agent at early and late stage of HCC development, in virtue of its ability to induce HCC regression *via* a re-differentiation program of neoplastic hepatocytes towards a more benign phenotype.

To our knowledge, this is the first evidence of a treatment able to reduce the incidence of HCC even when it begins at a time when HCC is already developed. This makes T3 a potential therapeutic agent in HCC therapy. Unfortunately, its clinical use is hampered by the several negative side effects exerted by this hormone, in particular cardiac dysfunctions [201].

Nevertheless, several novel TH analogs, such as GC-1 and KB2114 have recently been developed with the goal of generating effective and safe treatments to reduce T3-induced adverse side effects.

Thus, the possibility that these thyromimetic can provide a promising tool in HCC therapy can be envisaged.

Studies aimed at investigating whether these thyromimetics might recapitulate the anti-tumoral effects of T3 are already in progress in our laboratory.

15. FUTURE DIRECTIONS

The results presented in this work strongly support the anti-tumoral effect of T3 on early and late stages of hepatocarcinogenesis. Our experiments suggest that the anti-tumoral effect of T3 is associated with its ability to induce the reprogramming of pre/neoplastic hepatocytes towards a more mature and differentiated phenotype. Future work will be aimed at improving our knowledge about the molecular mechanisms driven by the hormone in *in vivo* and *in vitro* conditions.

Previous work from our laboratory showed a downregulation of the thyroid hormone receptor β in preneoplastic lesions and HCC in rats as well as in humans[169]. It would be important to understand whether the reprogramming of the undifferentiated hepatocytes at different stages of hepatocarcinogenesis is strictly associated with the presence of the receptor and the re-establishment of the T3/TR axis.

To explore this aspect, we are planning experiments in which T3 will be given to immunodepressed mice previously inoculated with human HCC cells transfected with THRβ.

In addition, since our results show an upregulation of KLF9 following T3 treatment, we will investigate whether activation of this transcription factors is necessary to induce the differentiation program. To this aim, we will knock out KLF9 in human HCC cells overexpressing THRβ, with and without T3 administration. The tumorigenic capacity of KLF9 silenced HCC cells will be investigated following inoculation of these cells in immunodepressed mice.

We also plan to extend our findings to selective agonists of THRβ. Indeed, it is well known that the therapeutic use of T3 is hampered by the cardiac negative side effects due to its interaction with the THRα receptor expressed in the heart.

Recently, new thyromimetics able to reproduce the beneficial effects of T3, in the absence of the undesired ones, have been developed. Therefore, we will make use of these THRβ agonists to reproduce the anti-tumoral effect of T3. It is important to mention that KB2115 (also known as Eprotirome) recapitulates the well-established hepatomitogenic activity of T3 without overt toxicity, suggesting that this agent may also be useful for regenerative therapies in liver

transplantation [125]. Moreover, another thyromimetic, GC-1 (Sobetirome) has been reported to exert a notable antitumoral effect on hMet-S45Y-β-catenin HCC obtained in FVB mice [137].

Based on this premise, a better knowledge of the new thyroid hormone analogs might lead to new strategies in HCC therapy based on their ability to induce a differentiation program in transformed hepatocytes.

16. REFERENCES

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