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DOTTORATO DI RICERCA

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TERAPIE AVANZATE BIOMEDICHE E CHIRURGICHE

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ciclo XXXV

The intrahepatic dysregulation of thyroid hormone signalling in liver carcinogenesis

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1. INTRODUCTION

1.1 Hepatocellular carcinoma

Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide, with approximately 906.000 new cases and 830.000 deaths [1]. Hepatocellular carcinoma (HCC) represents about 75%-85% of cases and its incidence is predicted to rise in the next years [2]. HCC predominantly arises on cirrhotic liver through the development of inflammation and fibrosis, predisposing the liver to dysplasia and subsequent malignant transformation [3].

The mechanisms involved in liver carcinogenesis are several and complex, with multiple genetic and epigenetic events resulting in gene rearrangements, somatic mutations and growth factors pathway alterations leading to tumor development and progression [4] [5]. The predominant molecular pathways involved in HCC pathogenesis include [3]:

- Activation of receptor tyrosine kinases: Ras/Raf/MEK/ERK and P13K/AKT/mTOR cascades
- Pathways regulating growth factor signalling, such as Insulin-like Growth Factor (IGF), Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Transforming-Beta Growth Factor (TGF-B), Hepatocyte Growth Factor (HGF/MET);
- Pathways related to cell differentiation: Wnt/β-catenin, Hedgehog, and Notch pathways.

- Pathways related to angiogenesis: vascular endothelial growth factor (VEGF) and FGF pathways

Despite the advances in this field, the molecular pathogenesis of HCC is not fully understood and, as consequence, useful biomarkers to early non-invasive diagnosis and to prognostic evaluation are lacking and current therapeutic options for HCC remain unsatisfactory.

1.2 Thyroid hormone signalling in liver diseases and carcinogenesis

Thyroid hormones (TH) influence a variety of physiological and pathological processes, such as development, metabolism, cell growth and proliferation [6]. Several studies have suggested a role of TH signalling in liver disease and emerging reports suggested a relationship between a dysregulation of TH homeostasis and hepatocarcinogenesis [7].

1.2.1 Molecular basis of TH action

Thyroid hormones (THs), namely 3,5,3'-triiodo-L-thyronine (T3) and 3,5,3',5' tetraiodo-L-thyronine (T4) are pleiotropic molecules that control different biological process. The primary circulating thyroid hormone, T4 (the prohormone), is deiodinated within cells by iodothyronine deiodinases type I and type II (D1, D2) to become biologically active T3. In contrast, deiodinase type III (D3) reduces intracellular thyroid activity by degrading T4 and T2 into the "inactive" metabolites reverse T3 (rT3) and T2, respectively. The expression

of deiodinase enzymes is modulated at different levels in response to serum fluctuation of T3 and T4 as well to a wide variety of intracellular signals. On entering the nucleus, the gene-regulating activity of T3 is mediated by binding to specific DNA sequences, known as thyroid hormone response elements (TREs), located on the promoter regions of thyroid hormone target genes [6].

T3 is responsible of the physiological and genomic effects of TH by binding to its nuclear receptors (Thyroid receptor TRα and TRβ) which are ligand-inducible transcription factors able to activating or repressing target genes [8]. The thyroid receptors (TRs) have tissue-specific distribution. While TRβ mediates the metabolic actions of T3 with a key role in the control of the lipid metabolism and is the known major receptor isoform expressed in the liver, TRα is expressed predominantly in the heart, skeletal muscle, and adipose tissues, and specifically mediates adaptive thermogenesis [9].

Membrane transporters such as monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), organic-anion-transporting polypeptide 1 (OATP1) transport TH into cells. Unbound TR may heterodimerize with retinoid X receptor (RXR), which then binds to a TRE and a corepressor complex. T3-binding to the ligand binding domain results in disruption of co-repression binding and promotes coactivator binding, which leads to positively regulated gene transcription. In absence of T3, corepressors may act to repress positively regulated genes and activate negatively regulated genes [6] [10] (**Figure 1**).

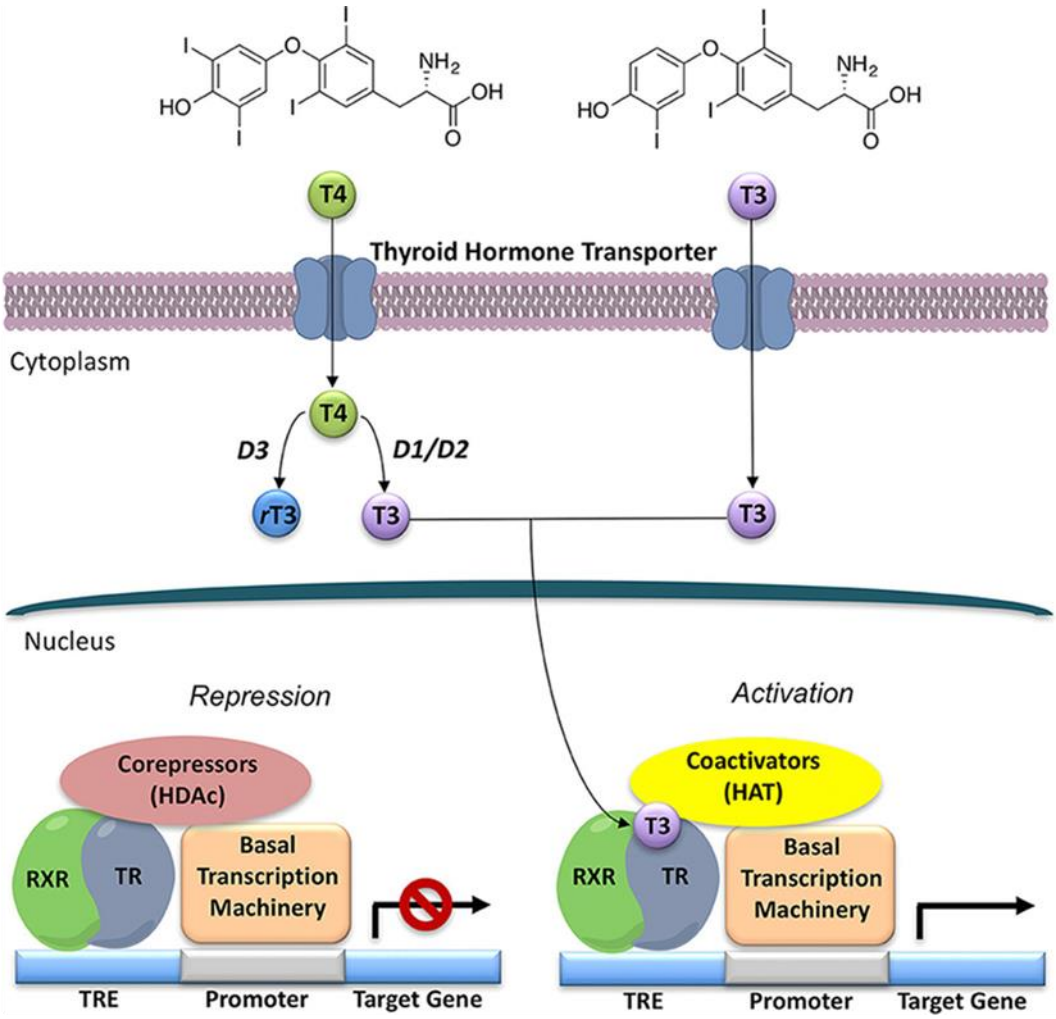

Figure 1. Genomic action of TH [11]

1.2.2 TH role in chronic liver disease

TH is a major regulator of lipid metabolism and modulates hepatic insulin sensitivity. Among individuals with non-alcoholic fatty liver disease (NAFLD), the prevalence of hypothyroidism is reported to range between 15.2% and 36.3% [12]. A population-based study reported that the prevalence of NAFLD and elevated alanine aminotransferase (ALT) is higher among patients with hypothyroidism [13]. Further evidence supporting the association

between the severity of chronic liver disease and hypothyroidism is provided by a larger population-based, prospective, cohort study showing that elevated T4 levels were associated with a lower risk of NAFLD, while higher TSH levels were associated with an increased risk of liver fibrosis. Intriguingly, NAFLD risk decreased when TH levels increased (i.e. from hypothyroid state to hyperthyroid state) [14]. On the basis of these observations, a new drug, Resmetirom, was developed. It is a liver-directed, orally active, selective thyroid hormone receptor-β agonist designed to improve NASH by increasing hepatic fat metabolism and reducing lipotoxicity. Advanced phase III clinical trial is ongoing with promising results [15].

Differences in TH levels have also been described for other chronic liver diseases. For example, hypothyroidism is more common among those with chronic HCV compared to healthy individuals, and higher TSH levels are also more common among those with advanced liver fibrosis [16] [17].

1.2.3 TH role in liver carcinogenesis

TH homeostasis and the associated signalling pathways have been shown to influence liver fibrogenesis and carcinogenesis (**Figure 2**).

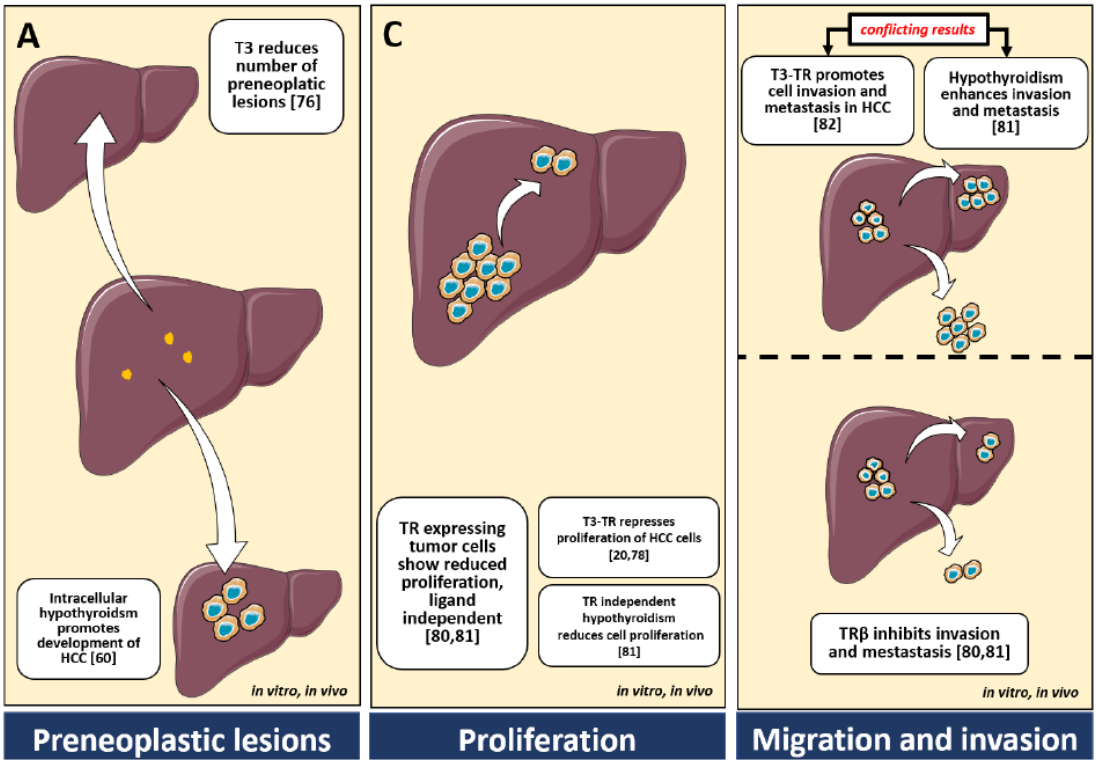

Figure 2. Effect of TH on different pattern of HCC [7].

Both systemic and local hypothyroidism were associated with HCC development [18]. Intrahepatic hypothyroidism could be a physiological response to liver injury, suggesting an intrinsic regulation of TH concentration in the liver tissue. For example, the HCC development was associated to an increase of rT3 levels, probably due to the D3 upregulation [19]. D3 is an oncofoetal protein highly expressed in embryonic tissue and in placenta and also detected in neoplastic tissues, playing a crucial role in human carcinogenesis [20]. In fact, in adult life its expression ceases and is switched on upon tumoral transformation, while remaining silent in the normal counterpart tissues. D3 proliferative effects have been associated with the Hedgehog signalling

pathway involved in several malignancies (up to 25% of human tumours), between activation of zinc-finger transcription factors Gli 2 [21]. During liver injury, D3 is upregulated, resulting in an increased hepatocyte proliferation; in contrast, D1 levels decrease [22]. The combination of a high D3 and low D1 results in low T3 and high rT3 which are conditions observed during critical illness. These observations suggest that biochemical hypothyroidism may be a normal physiological response to liver injury. As tumor (or HCC) growth evokes similar responses to development and injury, it is plausible that a hypothyroid state could favor cancer cell survival, proliferation, and differentiation [23].

Moreover, systemic hypothyroidism was associated with an increasing risk of HCC in women with a long history (>10 years) of thyroid disease [24]. In this setting, a recent study showed that, in a rat model, the T3 administration impaired HCC progression, even in advanced stages. Indeed, the TH administration induced a rapid differentiation reprogramming in hepatic preneoplastic lesions after 4 days of treatment compared with untreated rats. Remarkably, T3 treatment caused activation of pathways and transcription factors related to acquisition and maintenance of a differentiated phenotype; among these T3-activated transcription factors, a Kruppel-like factor (KLF9), which contains a TRE, is implicated in the regulation balance between cell-pluripotency, differentiation, and metabolism. Furthermore, increased D1 expression was revealed in addition to metabolic shift from glycolysis to oxidative phosphorylation [25].

However, the impact of T3 on HCC cancer progression and invasiveness remains controversial. Interestingly, administration of T3 in cell lines expressing endogenous TR promotes the invasive and metastatic potential of hepatoma cells through the activation of furin expression, leading to activation of matrix metallo-proteasis (MMPs) which consequently results in higher metastasis rate due to release of collagen fibres that facilitates the invasion of the tumor [26]; on the other hand, TRβ-expressing HCC shows a reduced tumor growth (evaluating the cell proliferation marker Ki-67), less vascularization and less mesenchymal phenotype. Due to these conflicting results, it remains unclear if unbound TRβ has a ligand-independent impact on metastasis and invasiveness, pointing out the role of tumor microenvironment in this setting.

1.2.4 TH status and liver microenvironment

Changes in the stromal cells secondary to intrahepatic hypothyroidism could modulate the cancer progression and metastatic growth independently of the TRβ expression in tumor cells [27]. Recent findings suggest the possible influence of TH on TGF-β signalling and Wnt/β-catenin pathway in stromal cell activation [28] [29].

Moreover, an association was found between changes in intrahepatic TH homeostasis and Hedgehog (Hh) signalling. Hh is a developmental morphogen implicated also in liver regeneration and fibrosis. Indeed, in the injured hepatocytes, Hh ligands activated hepatic stellate cells, progenitor cells, Kupffer cells, natural killer T cells and endothelial cells [30]. In rats and human fibrotic

liver tissues, decreased levels of D1 in hepatocytes were found, whereas an upregulation of D3 was observed in stromal cells. These changes seem to be regulated by Hh ligands. Furthermore, stromal cells undergo dedifferentiation during chronic injury from epithelial to a more mesenchymal activated phenotype, which is associated to higher invasive and metastatic potential [31].

In accordance with the aforementioned findings, there are recent insights into Hh-TH-D3 crosstalk from murine skin cancer models. In basal cell carcinoma (BCC), T3 inactivation by D3 seems to have a central role in the progression of the tumor, and D3 expression is regulated by Hh ligands including Sonic hedgehog (Shh). A direct induction of D3 by Shh/Gli2 has been shown in proliferating keratinocytes. This leads to reduce active TH levels concentrations, with increased cyclin D1 and keratinocytes proliferation. Furthermore, D3 depletion or T3 treatment induces apoptosis of BCC cancer cells attenuating the Shh signalling via a direct impairment of Gli2 protein by T3 trough PKA induction [21].

In conclusion, there are many evidence about the impact of TH on chronic liver disease-HCC axis, and still conflicting results are reported on development, proliferation and cancer migration. Other studies are needed to define accurate expression of TH regulator factors in HCC settings and the following clinical implications.

2. AIM

The aim of the study is to determine the role of TH signalling and its expression in liver carcinogenesis. Whit this objective, we will evaluate the role of deiodinases and their pathways in neoplastic liver tissue, in comparison with cirrhotic liver tissue and healthy liver tissue, investigating their impact on carcinogenesis and tumor progression.

3. METHODS

3.1 Study design and population

This is a proof of knowledge introductory to an ongoing monocentric prospective case-control study developed with the collaboration of Disease of the Liver and Biliary System Unit, Endocrinology Unit and Hepato-biliary Surgery Unit of the University of Naples "Federico II".

The protocol was approved by the local ethic board of Federico II Academic Hospital (n. 114/19). All patients and controls involved in the study provided written informed consent to participate to the study and for the scientific use of their tissue samples.

From November 2019 to December 2020, we enrolled 19 patients, who underwent liver surgery for HCC (cases) or for other non-neoplastic liver diseases in non-cirrhotic context (controls).

Data collection

The following clinical data were collected for all patients included in the study:

- Date of birth, sex, Body Mass Index (BMI)
- Indication to hepatobiliary surgical procedure
- Liver disease aetiology (e.g. alcoholic liver disease, non- alcoholic steatohepatitis, chronic hepatitis B or C)

- Prognostic assessment of chronic liver disease according to Child-Pugh score *for cirrhotic patients*
- HCC staging according to BCLC classification *for HCC patients*
- Liver stiffness with Transient Elastography (TE) by Fibroscan® recorded before the development of HCC *for cirrhotic patients*
- Assessment of clinically significant portal hypertension for *cirrhotic patients*
- Liver function and inflammatory tests (e.g. ALT, AST, GGT, ALP, albumin, INR, bilirubin)
- Serum cancer biomarker levels (e.g. AFP)
- Thyroid biochemical status

Survival Analysis

Patients' follow-up started from the liver resection, evaluating clinical outcomes for the neoplastic disease. The main clinical outcomes evaluated were Overall Survival (OS) and Progression-free Survival (PFS). OS was calculated from the liver resection to death. PFS was calculated from liver resection to HCC recurrence.

3.2 *Liver samples and histology*

Patients' liver samples were collected during hepatobiliary surgery. For HCC patients, samples were collected from HCC and from surrounding

cirrhotic liver. Liver samples of about 25-70 mg of weight were thus quickly frozen (within an hour) at -80°C temperature and then stored at -80°C.

For each patient, the liver tissue specimen was fixed, processed, and stained using standard hematoxylin and eosin. The resected liver parenchyma was analysed by expert liver pathologists regarding the presence of acute or chronic liver disease, the stage of fibrosis, cirrhosis, steatohepatitis for all patients. For HCC patients' evaluation of tumor grading (accordingly to Edmondson Classification) and staging was performed.

3.3 Western blot analysis

Western blot analysis was performed to evaluate the expression of iodothyronine deiodinase 1 and 3 (D1 and D3) in all tissue samples.

Total protein extracts from liver samples were prepared by using the following lysis buffer: TritonX-100, 25 mM TrisHCl pH 7,4, 300 mM NaCl, 1mM $CaCl_2$, protease inhibitors (Sigma), β- glycerophosphate, NaVa 0,2 M, NaF 20X, PMSF 200 mM. Protein concentration was measured with Biorad assay (Protein Assay, Biorad). Proteins were run on a 10% SDS-PAGE gel and transferred on PVDF membrane (Amersham Hybond-P). The membrane was then blocked with 5% non-fat dry milk in PBS, probed with anti-Dio1 and anti-Dio3antibodies, then washed with 1X PSB-0,2% Tween and incubated with HRP-conjugated donkey anti-rabbit IgG secondary antibody (1:3000), and detected by chemiluminescence (Biorad). After extensive washing, the membrane was incubated with anti-GAPDH-specific antibodies (1:5000, E-AB-

20059; Elabscience) as loading control. All Western blots were run in triplicate, and bands were quantitated in 1 representative gel.

3.4 Real-time PCR analysis

For all samples, RT PCR analysis was performed to evaluate mRNA levels of the most relevant TH target genes. Specifically, we analysed mRNA expression of Iodothyronine Deiodinases-1 (D1) monocarboxylate transporter 8 (MCT8), thyroid hormone receptor α (THRα), thyroid hormone receptor β(THRβ), and Kruppel-like factor-9 (KLF-9).

Total RNAs were extracted from liver samples with Trizol (Life Technologies, Ltd) according to manufacturer protocol, and retrotranscribed with Lunascript RT SuperMix (New England BioLabs). Genes expression was measured with Real-Time PCR using SYBR Green with iQ5 Multicolor Real Time Detector System (BioRad, Hercules CA). Primers have been designed to work under same conditions (95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min) generating products of comparable dimensions. For every reaction, cDNAs were diluted 5 times and samples were run in triplicate. Template concentration was determined from cycles number obtained during exponential phase of PCR reaction, at the threshold point (Ct). Genes relative quantities were calculated using GAPDH gene as internal control. Results were determined as following: N target= 2(Ct sample – Ct control). All Real-Time PCR experiments were run in triplicate.

3.5 Statistical analysis

The results of these analyses were correlated to the patients' clinicopathological characteristics. Normally distributed and skewed continuous variables were compared using Student's t-test and Mann-Whitney U test, respectively, described as mean ± standard deviation (SD) or median ± interquartile range (IQR). The categorical variable was described as proportion using Fisher's exact test to compare. Survival curves were estimated using the Kaplan-Meier method, and the differences in survival rates between groups were compared using the Log rank test. For all tests, a p-value < 0.05 was considered statistically significant. Data analysis and graphing were performed using SPSS Statistic (version 26.0, USA) and R language version 4.1.3.

Due to the descriptive and exploratory nature of the study, the sample size is not limited and therefore not chosen on the basis of power calculations. It is rather based on the experience from other studies including patients with liver resection for various diseases.

4. RESULTS

4.1 Study population characteristics'

We obtained HCC tissue, cirrhotic tissue and healthy liver tissue samples from 19 patients (12 males and 7 females, median age 63 (IQR 56-72) years undergoing liver resection.

In particular, we enrolled 10 (52,6%) patients affected by HCC developed on liver cirrhosis and 9 (47,4%) non-cirrhotic patients undergoing liver surgery for liver metastases from colon cancer, hepatic adenoma or echinococcosis.

Patients' clinical and biochemical characteristics are described in **Table 1**.

The two populations were different only for age and sex distribution. In fact, a more advanced age has been observed in HCC patient, together with an higher prevalence in male sex, as expected for the epidemiology of the tumor. Mean age in HCC cohort was 71.2±7.2 years with a male/female ratio 9:1.

The aetiology of underlying liver cirrhosis was mostly viral: HCV infection was predominant (70%), compared with HBV chronic infection (10%) and metabolic associated fatty liver disease (20%).

Overall, patients and controls did not show significantly different characteristics for the main liver function parameters.

Median basal liver stiffness measurement for cirrhotic patients performed with TE by Fibroscan before development of HCC was 32 (IQR 23.47-35.5) kPa.

All HCC patients had underlying compensated liver cirrhosis according to Child Pugh score A5 and MELD score < 10. Clinically significant portal hypertension was diagnosed in 40% of cirrhotic patients.

Prognostic stratification of HCC patients was made accordingly to the Barcelona Clinic staging system (BCLC). All HCC patients showed an early-stage disease (BCLC A).

The baseline characteristics of HCC patients are described in **Table 2.**

4.2 TH metabolism

We evaluated the genes and protein expression of TH metabolism genes with quantitative reverse transcription PCR analysis (RT-PCR) and Western Blot analysis, respectively.

RT-PCR Analysis

Results of mRNA expression of these genes were measured by quantitative reverse transcription PCR analysis as shown in **Figure 3**.

Iodothyronine Deiodinases-1 (DIO1) mRNA expression decreased gradually together with the expression of the monocarboxylate transporter 8 (MCT8) and thyroid hormone receptor α (THRα) in cirrhotic and HCC samples compared to controls. Although non statistically significant, we observed also a gradually decreasing trend of thyroid hormone receptor β (THRβ) from controls to HCC.

Furthermore, we observed that Kruppel-like factor-9 (KLF-9) expression, a TH target gene implicated in the regulation of the balance between pluripotency,

differentiation, and cell metabolism, decreased in HCC patients compared to controls.

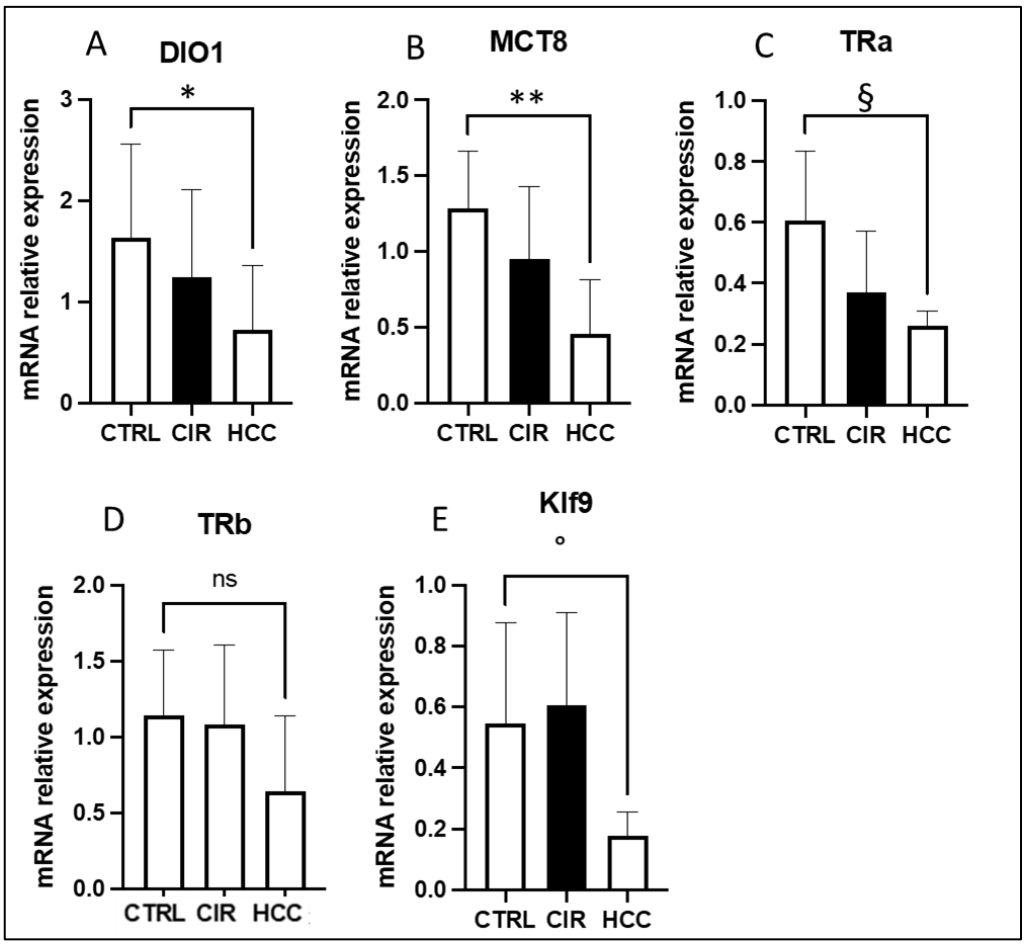

Figure 3. Real-time PCR analysis of mRNA expression of TH target genes. DIO1 (A), MCT8 (B), ThRα (C) levels decreased gradually from healthy liver (controls, CTRL), to cirrhosis and HCC. Although non statistically significant, ThRb gene expression (D) showed a decreasing trend from controls to HCC. Level of KLF9, also decrease in HCC patients compared with controls (E). *p 0.04; **p 0.001; ° p 0.03; § p 0.02.

DIO 1: iodothyronine deiodinase 1; MCT 8: monocarboxylate transporter 8; TR: Thyroid hormone receptor; KLF 9: Kruppel like factor 9. CTRL: Controls; CIR: Cirrhotic; HCC: Hepatocellular Carcinoma.

Western Blot Analysis

Western blot analysis was performed to determine the protein expression of deiodinase type 1 and 3 in the three groups.

As shown in **Figure 4,** the protein DIO1 expression was decreased in HCC samples compared to controls. Conversely, D3 is absent in healthy samples and in cirrhotic liver, but is present in in 50% of HCC samples, thus demonstrating that in HCC the metabolism of TH is decreased compared to normal liver tissue.

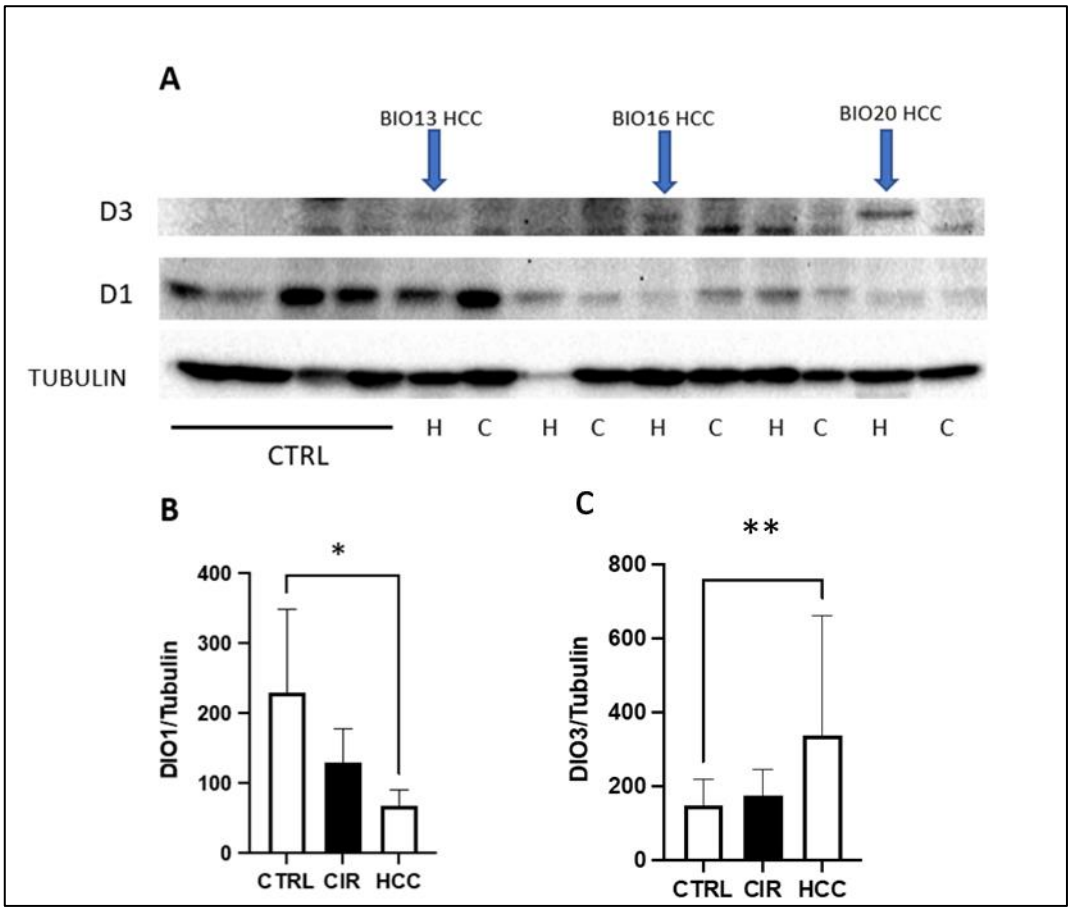

Figure 4. A. D1 and D3 expression were measured by Western blot analysis. The expression of D1 decreased in all patients with cirrhosis, while D3 is increased in 50% of HCC patients. B. DIO1 protein expression. *p 0.02. C. DIO3 protein expression **p 0.01.

4.3 Comparison of clinical characteristics in D3 positive and D3 negative HCC patients

Baseline clinical characteristics of D3 positive and D3 negative HCC patients were compared to determine whether the expression of D3 might distinguish a subgroup of HCC patient with specific clinical features (**Table 3**).

Among HCC patients, D3 expression is associated with increased liver stiffness (p=0.002) and BMI (p=0.004).

No statistically significant relationship was found with the presence of altered liver function and inflammatory tests, portal hypertension grade, and tumor burden, in terms of single or multiple HCC and tumor size, grading. Furthermore, no statistically significant relationship was found with alterations of thyroid status.

Median overall survival (OS) of D3 positive HCC patients was 17.2 months (IQR 13.7-24.8), while for D3 negative patients was 41.3 months (IQR 35.1-43.8).

The progression-free survival (PFS) was 15 months in D3 positive patients and 41.3 months in D3 negative patients. HCC recurrence was observed in 60% of D3 positive patients' ad in 20% of D3 negative patients.

Among D3 positive HCC patients, we found that 3 patients died for liver disease progression and 1 patient died for cerebral haemorrhage.

Although not statistically significant, we observed an increased frequency of death in D3 positive patients, suggesting that D3 expression could be associated with a worse prognosis compared to D3 negative HCC patients.

Kaplan-Meyer analysis reported a statistically significant OS difference between the two groups (log rank p 0.003), with a median OS of 17.9 (15.5-18.7) months for D3 positive HCC patients (**Figure 5**).

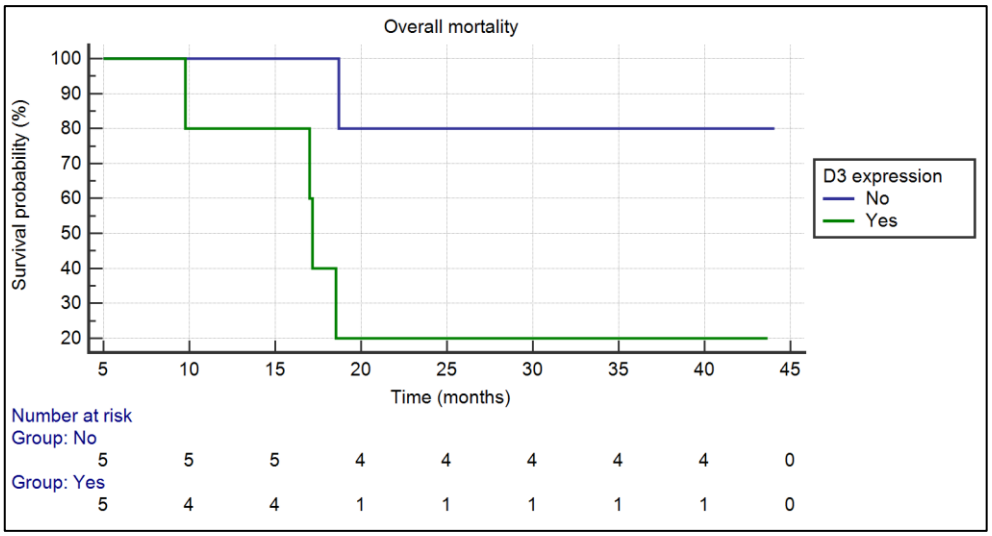

Figure 5. Kaplan-Meyer survival curve in HCC patients D3+ and D3-.

5. DISCUSSION

Systemic and local hypothyroidism have been associated with HCC development and progression. Liver is a known target organ of TH and the effects on cellular proliferation and differentiation are driven by TRs, which are known factors implicated in hepatocarcinogenesis [25]. Although it has been proven that the expression of D1 and D3 change during induced liver injury [7] [22], in HCC setting, the role of deiodinases is still poorly understood.

In this preliminary study we investigated the expression of TH regulator factors in HCC tissue samples, in cirrhotic liver surrounding HCC samples and in healthy liver tissue samples evaluating the difference of expression of these factors in the three groups.

The principal results of our study showed that:

- D1 decreases both in cirrhotic liver and HCC samples, compared with controls, confirming the down-regulation of D1 during liver injury;
- D1 decreased expression is associated with a concomitant decrease in transporter molecules expression, such as MCT8 and TRs. Furthermore, these factors expression decreased gradually from healthy liver to HCC;
- KLF9 mRNA decreased expression was observed in parallel with reduced TR expression;
- D3 mRNA expression increased in half of HCCs, while it was not found in cirrhotic samples.

These findings confirmed the status of intrahepatic hypothyroidism in HCC described in literature during acute and chronic liver injury and in preneoplastic lesions [25], thus confirming the independent regulation of TH in liver. The novelty of this study is represented by the fact that for the first time it was reported that HCC tissue presents a more severe TH intrahepatic dysregulation compared with the surrounding liver cirrhotic tissue.

Moreover, we compared clinical characteristics of D3 positive HCC patients with D3 negative HCC patients. Of note, thyroid diseases were not associated with the increase of D3 in HCC samples, thus emphasizing the intrinsic intrahepatic TH regulation during liver injury and hepatocarcinogenesis, which is independent from TH serum concentration.[8]

Liver stiffness measurement, as a marker of fibrosis, was significantly higher in D3 positive HCC compared with D3 negative HCC patients. This data is in line with the study of *Bohinc BN. et al*. that confirmed the hypothesis that the injured liver is in a state of intrahepatic hypothyroidism, identifying a role for hepatic stromal cells and the canonical Hedgehog pathway as key regulators of TH homeostasis during adult liver injury [31]. Hence D3 expression could be a hallmark of more severe fibrosis and could become a candidate molecule as prognostic factor for HCC behavior. On this point, we are going to study the expression of D3 protein by immunohistochemical analysis to define the characteristic cellular

distribution (epithelial compartment or stromal compartment as expected from our preliminary results).

In fact, in other tumor models, such as colorectal cancer, deiodinases specific-cell compartment distribution was defined. In colorectal cancer, D3 protein expression, analyzed by immunohistochemistry, was highly expressed in the epithelial compartment of adenomas and colon carcinomas, while it was nearly absent in the stromal component [32].

Decreased expression of TRs and KLF-9 was also reported by *Kowalic MA. et al*, showing that, in rat models, oral T3 administration induces a genomic reprogramming in preneoplastic liver cells through the activation of pro-differentiating transcription factors, including KLF9 [25].

Furthermore, we observed an association between D3 positivity and BMI in patients with HCC. However, the median BMI of patients from both groups is representative of overweight subjects. Thus, it is possible that a possible association with liver steatosis did not emerge from our data. Though, due to the lack of data describing this association in literature, it is not possible to interpret this data.

Hepatic mortality showed only a trend between the two groups (D3+ and D3-) Therefore, we performed a Kaplan-Meyer survival analysis, which reported a significantly difference between survival over time (log rank 0.03), with a reduced survival time among D3+ patients. This data seems to be not related to grading, staging, burden and recurrence of the tumor.

The finding of a lower survival probability in patients with D3 may be explained with a more aggressive phenotype of the tumor, but further studies are needed. On the basis of this preliminary data, we think that D3 positivity could be considered a negative prognostic factor in the setting of HCC, in terms of predicting the HCC development and tumor behaviour.

Studies carried out on other tumor models enhanced the deiodinases' implication in tumor development and progression with unclear results. In the setting of colorectal cancer, *Dentice M. et al*. showed that the D3 expression was upregulated in tumorigenesis but negatively correlated with the histological grading and tumor progression; in contrast D2 expression decreased in this phase, but its role remains poorly understood [32].

Furthermore, in non-melanoma skin cancer D3 rapidly increased during the initial tumorigenesis step with higher expression up to the formation of papillomas, while D2 increased expression was found at the later phases of tumorigenesis with high impact on cancer invasiveness [33].

On the basis of these results, the role of D2 in HCC setting could be further investigated, but a larger sample size is needed.

Though, this study has some limitations. The sample size is small, though homogeneous for sex and HCC patients were not selected for liver disease aetiology.

For its nature of proof of knowledge, this study could help us to define the potential direction of further studies, also considering that there are only few and incomplete studies on this matter in literature.

6. CONCLUSIONS

The impact of TH signalling in HCC development and progression is still poorly understood. With these preliminary data we conclude that D3 positivity in HCC patients could define a more severe phenotype of HCC and it could be used in clinical practice as negative prognostic indicator of HCC development and progression. Our findings need to be applied to a larger sample size to be confirmed.

7. TABLES

Table 1. Baseline characteristics of patients

Parameter	**Overall (N 19)**	**HCC (N 10)**	**Non cirrhotic patients* (N 9)**	**p value**
N (%)	19 (100)	10 (52.6)	9 (47.4)	-
Sex, n (%) *M* *F*	 12 (63.2) 7 (36.8)	 9 (47.3) 1 (5.3)	 3 (15.8) 6 (31.6)	**0.02**
Age, years, median (IQR)	63 (56.8-71.8)	71.5 (69-71)	56 (53.7-60.7)	**0.002**
BMI, Kg/m^2, median (IQR)	25.3 (23.9-27.7)	28.8 (25.75-29.5)	24.9 (23.5-26)	**0.049**
Thyroid disease, yes, n (%)	5 (26.3)	2 (10.5)	3 (15.8)	0.521
Albumin, g/dl, median (IQR)	4.4 (4-4.6)	4.4 (4-4.62)	4.4 (4-4.6)	0.120
Total bilirubin, mg/dl, median (IQR)	0.7 (0.43-1.1)	0.75 (0.47-1.25)	0.7 (0.41-1.2)	0.153
INR, IU/L, median (IQR)	1.02 (0.9-1.1)	1.07 (0.97-1.12)	0.97 (0.85-1.1)	0.691
AST, IU/L, median (IQR)	22 (18-31)	22 (18.5-31)	28 (18-29)	0.653
ALT, IU/L, median (IQR)	25.5 (21-30)	25 (19.75-32.25)	25.5 (24-27)	0.8125
GGT, IU/L, median (IQR)	41.5 (23.2-103)	46 (20-115.5)	41.5 (29.7-134.8)	0.457
ALP, IU/L, median (IQR)	84.5 (71.7-127.3)	78.5 (69.7-121.3)	101 (74.7-147.8)	0.326

Abbreviations: BMI: Body Mass Index; INR: International Normalized Ratio; AST: Aspartate Aminotransferase, ALT Alanine aminotransferase, GGT: gammaglutamil-transpeptidase; ALP: alkaline phosphatase.

***Non cirrhotic patients**: 7 patients underwent liver surgery for liver metastases from colon cancer; 1 patient underwent liver surgery for echinococchosis, 1 patient underwent liver surgery for hepatic adenoma

Table 2. Baseline characteristics of HCC patients.

	N	**%**
Cirrhosis	10	100
Aetiology		
HCV	7	70
HBV	1	10
MAFLD	1	10
VIRAL+MAFLD	1	10
Portal hypertension	4	40
Tumor burden		
Single nodule	7	70
Multiple nodules	3	30
Grading sec Edmondson		
G1	2	20
G2	4	40
G3	4	40

Abbreviations: HCC: Hepatocellular carcinoma; HCV: Hepatitis C Virus; HBV: Hepatitis B virus; MAFLD: Metabolic associated Fatty Liver Disease

Table 3. Comparison of clinical characteristics in D3 positive and D3 negative HCC patients.

Parameter	**D3+ (N 5)**	**D3- (N 5)**	**p value**
Sex, M, n (%)	5 (100)	4 (80)	1
Age, years, median (IQR)	69 (67-72.7)	74 (68.7-78.2)	0.346
BMI, kg/m^2,median (IQR)	29.5 (26.6-31.6)	25.1 (23-25.5)	0.046
Thyroid disease, yes, n (%)	0	1 (20)	1
Albumin, g/dl, median (IQR)	4.4 (3.9-4.6)	4.4 (4.2-4.6)	0.753
Total bilirubin, mg/dl, median (IQR)	0.8 (0.6-1.2)	0.5 (0.3-1.0)	0.291
INR, IU/L, median (IQR)	1.01 (0.9-1.1)	1.1 (1.0-1.1)	0.179
ALT, IU/L, median (IQR)	25 (16-30.7)	27 (23.2-48.2)	0.295
AST, IU/L, median (IQR)	31 (20.7-40.5)	20 (18-24.2)	0.246
ALP, IU/L median (IQR)	71 (63.7-75.2)	115 (87.7-151.5)	**0.0163**
GGT, IU/L, median (IQR)	55 (20-212)	37 (18.75-69)	0.503
AFP, ng/ml, median (IQR)	116 (5.7-187.5)	3.2 (2.6-429)	0.602
Liver Stiffness, kPa median (IQR)	32 (23.4-35.5)	14.1 (7.7-3.8)	**0.028**
Hepatic steatosis, yes, n (%)	2 (40)	3 (60)	1
Aetiology, n (%) *HCV* *HBV* *MAFLD* *HCV+MAFLD*	 4 (80) 0 0 1 (20)	 3 (60) 0 1 (20) 1 (20)	1
Portal hypertension, n (%)	3 (80)	1 (20)	0.5
Tumor burden, n (%) *Single nodule* *Multiple nodules*	 4 (80) 1 (20)	 3 (60) 2 (40)	1
BCLC A, n (%)	5 (100)	5 (100)	1
Grading sec Edmondson, n (%) *G 1* *G 2* *G 3*	 0 3 (60) 2 (40)	 2 (40) 2 (40) 1 (20)	 0.444 1 1
All-cause Death, n (%)	4 (80)	1 (20)	0.0719
Overall Survival, months, median (IQR)	17.2 (13.7-24.8)	41.3 (35.1-43.8)	0.059
HCC Recurrence, n (%)	3 (60)	1 (20)	0.167
Progression free survival, months, median (IQR)	15 (12.2-25.2)	41.3 (33.9-43.7)	0.143

Abbreviations: BMI: Body mass index; INR: International Normalized Ratio; ALT: Alanine aminotransferase AST: Aspartate Aminotransferase,; ALP: alkaline phosphatase; GGT: gammaglutamiltransferase; AFP: alphafetoprotein; BCLC: Barcelona Clinic Liver Cancer classification

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