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Faculté de médecine
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Chemoprevention of hepatocellular carcinoma
Proof of concept in animal models

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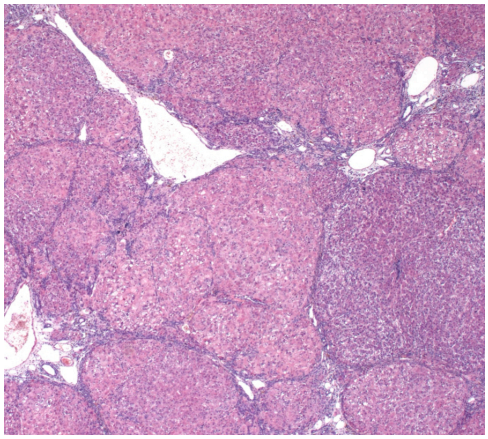

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List of abbreviations:

- 15D-PJG2: 15-deoxy-Δ12, 14-prostaglandin J2
- AAF: acetylaminofluorene
- ARF: alternate reading frame
- BCLC: Barcelona Clinic Liver Cancer
- CdK: cyclin-dependent kinase
- C/EBPα: CAAT enhancer binding protein alpha
- CKi: cyclin-dependent kinase inhibitor
- COX-2: cyclooxygenase-2
- CTL: control
- CYP: cytochrome p450
- DEN: diethylnitrosamine
- DNET: digestive neuro-endocrine tumours
- EGF: Endothelial growth factor
- EGFR: Epithelial growth factor receptor
- FAH: foci of altered hepatocytes
- GH: Growth Hormone
- GPE1: GST-p enhancer 1
- GSTp: glutathione S-transferase of the rat
- HCC: hepatocellular carcinoma
- HBV: hepatitis B virus
- HCV: Hepatitis C virus
- HSC: hepatic stellate cells
- HSCO: Hepatoma Subtracted-cDNA library Clone One
- HGDN: high grade dysplastic nodule
- Id-1: Inhibitor of differentiation/DNA binding protein 1
- IFN: interferon
- IGF-1: Insulin growth factor 1

- IGF2R: Insulin-like growth factor 2 receptor
- IHC: immunohistochemistry
- IND: induced
- LAN: lanreotide
- LCD: large cell dysplasia
- LGDN: low-grade dysplastic nodule
- Mdm2: Murine double minute 2
- MEK: Mitogen-activated protein kinase kinase
- mTOR; mammalian target of rapamycin
- NASH: non alcoholic steatohepatitis
- NFκB: Nuclear factor k B
- PDGF: platelet-derived growth factor
- PPARγ: peroxisome proliferator-actived receptor gamma
- PPRE: peroxisome proliferator response element
- PGZ: pioglitazone
- Pten: phosphatase and tensin homolog
- Rb: Retinoblastoma
- RCT: randomized controlled trial
- ROS: reactive oxygen species
- RXRα: retinoid X receptor alpha
- SMS: somatostatin
- sstr : somatostatin receptors
- TGFα : transforming growth factor α
- TGFβ : transforming growth factor β
- TZDs: thiazolidinediones
- VEGF: vascular endothelial growth factor
- WB: western-blot

Summary:

In the present work, we have evaluated the possibility of preventing liver carcinogenesis in rats at two stages of development. In the first series of experiments, we induced *foci of altered hepatocytes*, (FAH) which represent the first events in rodent liver carcinogenesis, using the chemical mutagens diethylnitrosamine (DEN) and acetylaminofluorene (AAF). In the second part of the work, we used repeated weekly injections of DEN only, that gave rise to significant fibrosis at 12 weeks and the development of malignant tumours at 16 weeks. We chose to assess the chemopreventive effect of two different drugs: pioglitazone and lanreotide. Pioglitazone is an agonist of PPARγ, itself a member of the nuclear receptor superfamily, responsible for the modulation of a number of metabolic pathways, including cell differentiation, metabolism of lipids and inflammation. The second drug used was lanreotide, a somatostatin analogue that has an inhibitory effect on the release of several hormones, such as growth hormone and serotonine.

We showed that both drugs were efficient in the first, pre-neoplastic model, by reducing the size of FAH, decreasing proliferation specifically in FAH by interacting with proteins of the cell cycle. We could also demonstrate that lanreotide increased apoptosis. In the second model, lanreotide was able to diminish the number of established hepatocellular carcinoma (HCC) by decreasing proliferation, in parallel with an anti-fibrotic action. Furthermore, enhanced apoptosis and anti-angiogenic effects were observed when lanreotide was given from the start of the carcinogenic induction by DEN. The cellular mechanisms leading to its effects warrant further investigations. On the contrary, pioglitazone did not prevent the appearance of neoplastic lesions. For these reasons, we did not analyse further its mechanism of action in the second model.

Altogether, the results we obtained demonstrate an activity of lanreotide, both at the early onset of liver carcinogenesis, and later on when advanced fibrosis, cirrhosis and HCC are induced.

Résumé:

Dans ce travail, nous avons évalué la possibilité de prévenir la carcinogenèse hépatocellulaire à deux stades de son développement chez des rats. Dans la première série d'expérimentations, nous avons induit des *foyers d'hépatocytes altérés* (FAH) qui représentent le premier stade de la carcinogenèse hépatique chez les rongeurs, moyennant l'utilisation de mutagènes chimiques, la diéthylnitrosamine (DEN) et l'acétylaminofluorène (AAF). Dans la seconde partie de notre travail, nous avons utilisé des injections répétées de DEN pour induire une fibrose significative en 12 semaines et le développement de tumeurs malignes en 16 semaines. Nous avons choisi de tester l'effet préventif de deux médicaments : la pioglitazone et le lanréotide. La pioglitazone est un agoniste des PPARγ, récepteurs nucléaires modulant de multiples voies métaboliques, tels la différentiation cellulaire, le métabolisme lipidique, l'inflammation. Le lanréotide est un analogue de la somatostatine ayant un effet inhibiteur sur la sécrétion de plusieurs hormones, comme l'hormone de croissance et la sérotonine.

Nous avons montré que les deux drogues sont efficaces dans le premier modèle (foyers pré-cancéreux) en réduisant la taille des FAH, par l'inhibition de la prolifération cellulaire spécifiquement dans les FAH et l'interaction avec les protéines du cycle cellulaire. Nous avons aussi pu montrer un effet pro-apoptotique du lanréotide. Dans le second modèle, le lanréotide a entrainé une diminution du nombre d'hépatocarcinomes, par un effet ant-prolifératif et anti-fibrotique. De plus, lorsqu'il était administré dès le début de l'expérimentation, nous avons pu observer un effet anti-angiogénique et pro-apoptotique. Les mécanismes sous-tendant ces effets doivent encore être explorés. Par contre, la pioglitazone n'a pas diminué l'incidence des nodules néoplasiques. Pour ces raisons nous n'avons pas poursuivi plus avant l'analyse de son action.

Au total, nous avons démontré un effet potentiel du lanréotide, en prévention du développement de lésions, tant prénéoplasiques que néoplasiques, lorsqu'une cirrhose est présente.

I. Introduction

A. *Hepatocellular carcinoma in 2009:*

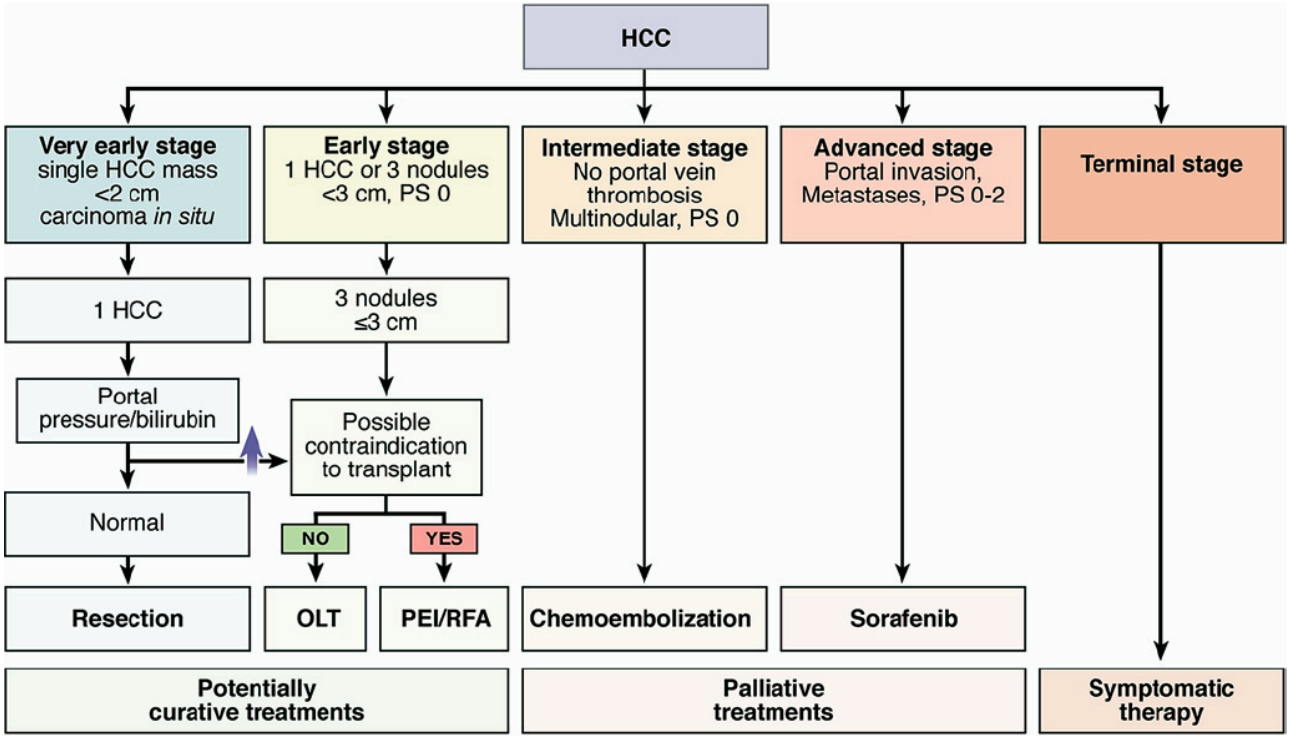
1. Epidemiology and risk factors

Hepatocellular carcinoma (HCC) is the fifth cancer in the world and the third most common cause of cancer mortality (1). Its distribution is highly heterogeneous around the globe, with areas of high incidence (from 17 to 90 cases/100.000 inhabitants/year), like China, southeast Asia, sub-Saharan Africa, where HCC is the most frequent cancer, and other regions, like Europe, Americas, where incidence is much lower (2-10/100.000 inhabitants/year). This important variation reflects the many different causal factors that can lead to HCC. The major risk factor for HCC is liver cirrhosis, but also advanced fibrosis. It is well known that cirrhosis itself is the consequence of chronic liver injury, mainly due to hepatitis B and C viruses, alcohol intake, but also diabetes mellitus and obesity (2). In high-incidence areas, the main cause of HCC is endemic exposure to hepatitis B virus (HBV). HBV is therefore recognized as the major risk factor of HCC, accounting for half of all HCC cases worldwide (3). In some areas of China and Africa, co-exposure to HBV and aflatoxinB1, a mycotoxin produced by *Aspergillus fungus,* recognized as a potent carcinogen, can increase the risk of HCC up to 60-fold (4). Hepatitis C virus (HCV) is the second most frequent cause of HCC, accounting for 30% of the cases (3) and is the main cause of HCC in developed countries, i.e. Europe, Japan, North America. Compared to HBV, HCV oncogenic properties are less studied. Most, patients being infected with HCV develop first cirrhosis then HCC. However, transgenic models of HCV-induced HCC showed that the HCV core protein is involved in oncogenesis. Authors demonstrated that HCV core transgenic mice overexpressed interleukin-1 and TNF, and that they produced Reactive Oxygen Species (ROS), even in the absence of inflammation (5).

Table 1: Child-Pugh Score

Points	*1*	*2*	*3*
Bilirubin (mg/dl)	<2	2-3	>3
Albumin (g/dl)	>3.5	2.8-3.5	>2.8
INR	<1.7	1.7-2.3	>2.3
Ascites	none	controlled	refractory
Encephalopathy	none	Grade I-II	Grade III-IV

Figure 1: Adapted BCLC classification, from (14)

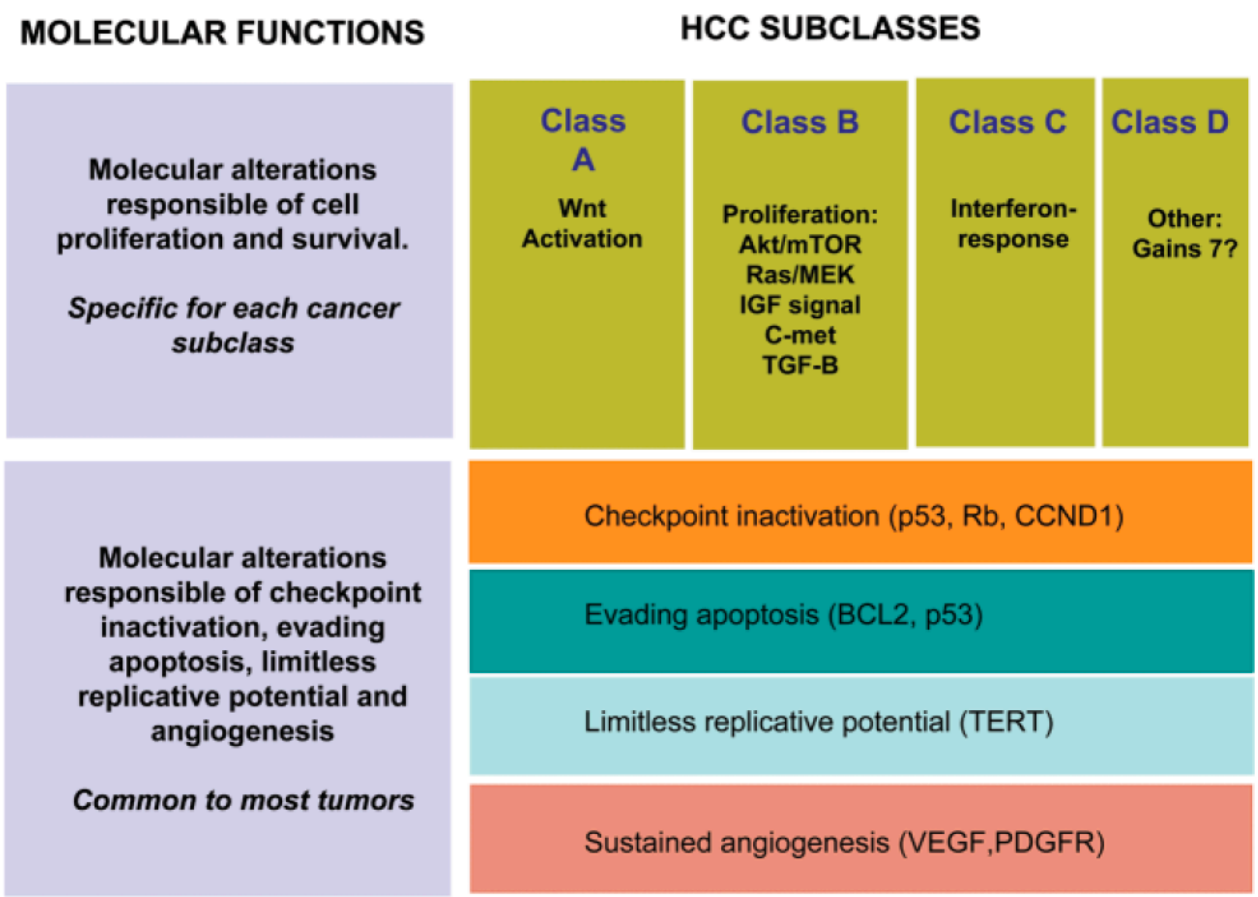


Other studies suggest that the core protein acts as Wnt ligand, thereby activating its pathway, leading HCC cells to proliferate (6). Alcohol is the third most frequent cause of HCC; it gives rise to cirrhosis, eventually leading to HCC, with 10% of HCC being attributable to alcohol abuse (7). Finally, tobacco, diabetes and obesity seem to be linked to increase in HCC incidence, but firm data to establish the strength of this association are still lacking (8-10).

2. Treatment options:

An important feature of HCC is that it develops, at least in Belgium and other European countries, in patients with underlying liver disease. This is a unique situation in oncology, as patient prognosis can be influenced either by liver function deterioration, or by tumour evolution. Liver function is generally classified according to the Child-Pugh score, with patients falling in 3 classes, A, B and C, from best to worse liver function (Table 1) (11). Available curative therapies are restricted to Child-Pugh class A patients. The worst the liver function, the less the therapeutic options, with the exception of liver transplantation, accessible to Child-Pugh B and C patients. It is therefore crucial to stage patients precisely for tumour status as well as for liver function. Several staging and prognostic scores have been proposed by different teams. The Barcelona Clinic Liver Cancer (BCLC) staging system seems to be the one that best selects patients for treatments and has been endorsed both by the American and European societies of hepatology (12). This system takes into account size and number of lesions, liver function according to Child-Pugh score and performance status according to WHO (13). It classifies patients in 5 categories: very early stage, early stage, intermediate stage, advanced stage and terminal stage (Figure 1). The first two categories bear the best prognosis and benefit from so-called curative options,

Figure 2: A proposal of molecular pathogenesis classification of HCC, from (18)



i.e. transplantation, surgical resection and percutaneous ablation. Intermediate stage comprise patients with well preserved liver function but multifocal intra-hepatic tumours, not candidate for transplantation, that will benefit from chemo-embolization (15). Until recently, there was no proven therapy for the BCLC advanced stage category, consisting of patients with vascular invasion and/or metastases but with still well preserved liver function. Sorafenib, a multi-kinase inhibitor, has shown to increase survival from 7.9 to 10.7 months in a placebo-controlled randomized phase III trial in this category of patients (16). Finally, Child-Pugh C patients developing HCC and who are not candidate for transplantation, with poor performance status (WHO grade >2), do not benefit from any kind of interventional therapy, and should be considered for best supportive care. At the time of diagnosis, curative treatments can be applied to only 25-30% of patients (17); intermediate and advanced stages represent another 50-60%, and terminal stage patients represent 15-20% of the whole HCC population, responsible for the poor prognosis of the disease (18, 19). There is thus room for more intense surveillance, leading to earlier diagnosis, thereby allowing shifting the treatment balance to more curative treatments.

Another way to classify HCC is through genomic analyses, an approach that is the object of research of many teams around the globe. Based on recent literature, Llovet and Bruix proposed a molecular classification (18) that is schematically represented in Figure 2. This classification stills evolves with recent findings and will eventually lead to identification of molecular targets for therapy, such as the mTOR or RAS pathways.

TABLE 2 RCT Investigating Adjuvant Effects of IFN After Resection or Ablation of HCC

Reference (year)	Intervention	IFN	IFN (n)	Control (n)	IFN Dose	HCV (n)	HBV (n)	HCV+ HBV (n)	Outcome
Ikeda et al[18] (2000)	Resection, n = 16; ethanol, n = 4	IFN-α	10	10	6 MIU 2× weekly for 36 mo	20	0	0	Early recurrence ↓
Kubo et al[19,20] (2001, 2002)	Resection	IFN-α	15	15	6 MIU daily for 2 wk, 3× weekly for 14 wk, and 2× for 88 wk	30	0	0	Late recurrence ↓ and improved long-term survival
Shiratori et al[21]	Ethanol	IFN-α	49	25	6 MIU 3× weekly for 48 mo	74	0	0	No impact on 1st recurrence but reduced 2nd and 3rd recurrence
Lin et al[22] (2004)	Ethanol	IFN-α	20	10	3 MIU 3× weekly every mo; 3 MIU daily for 10 d every mo	13	16	1	Late recurrence ↓ and improved 4-yr survival
Sun et al[23] (2006)	Resection	IFN-α	118	118	3 MIU 2× weekly for 2 wk followed by 5 MIU 3× weekly	0	236	0	Early recurrence ↓ and improved long-term survival
Mazaffero et al[25] (2006)	Resection	IFN-α	76	74	3 MIU 3× weekly for 48 wk	80	0	70	No effect on overall recurrence but late recurrence ↓ for pure HCV
Lo et al[24] (2007)	Resection	IFN-α	40	40	10 MIU/m^2 3× weekly for 16 wk	2	77	1	Early recurrence ↓ and improved 5-yr survival for stage III/IVA

From (25)

1. Current status on chemoprevention of HCC:

Several levels of prevention can be considered: first, primary prevention represents treating or preventing the diseases leading to cirrhosis; anti-viral treatments or vaccination are the main ways to achieve such prevention. Secondary prevention is the revention of the first occurrence of HCC once cirrhosis is established. Finally, tertiary prevention refers to treatments that aim at preventing a second occurrence of HCC after the curative treatment of a first tumour.

The strongest proof of concept that primary prevention is efficient is reflected by the national HBV vaccination campaign in Taiwan initiated in the 80's and reported in the New England Journal of Medicine (21), where 10 years after starting the vaccination of newborns, a significant decline of death due to HCC was observed in vaccinated children over 6 years. Evidence for secondary prevention in HBV-related HCC is less robust, with only one smaller study showing that treating patients with cirrhosis or advanced fibrosis using lamivudine, an antiviral medication, could also decrease HCC occurrence (22).

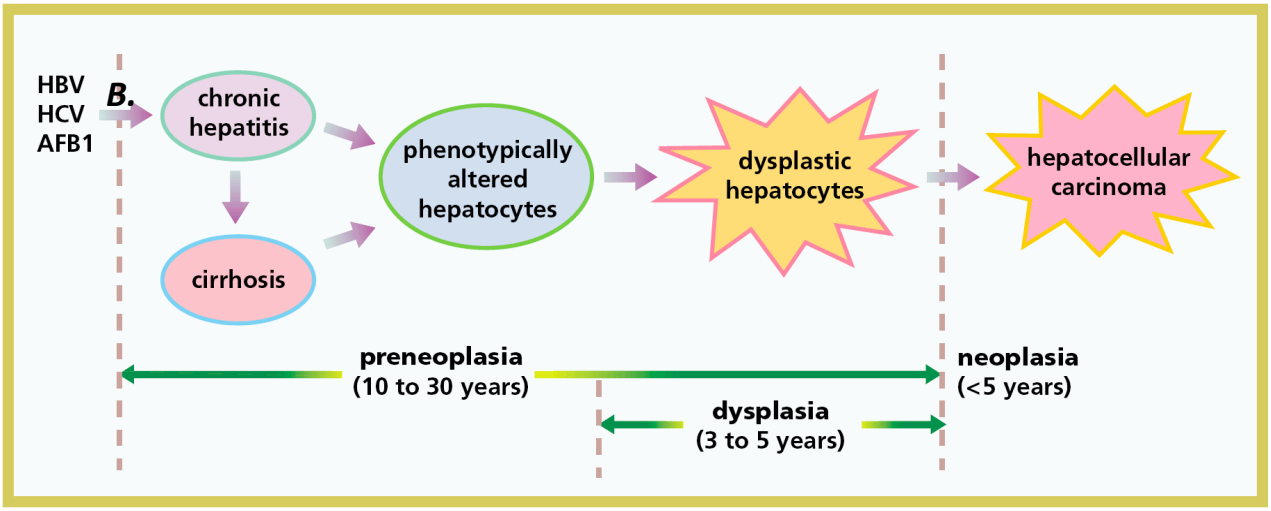
For patients with HCV-related HCC, there is no proof of evidence of efficacy of any treatment in primary prevention. In secondary prevention, a meta-analysis of 3 randomized controlled trials (RCT) and 15 non RCT showed a slight reduction in the occurrence of HCC in sustained responders under interferon (IFN) therapy (23), most of the studies coming from Japan. In tertiary prevention, a recent RCT showed a slight benefit on late recurrence, after resection of a first occurrence of HCC, in Caucasian patients with sustained response under IFN monotherapy (24). Results of all RCTs using interferon to prevent recurrence after resection or local ablation are shown in Table 2 (25). Recently, Lok et al. published a study addressing the potential effect of the pegylated form of IFN in preventing HCC occurrence in patients who did not have a response to anti-viral treatment. They failed to show a beneficial effect (26).

All these studies focused on IFN, which is well known for its anti-viral properties but has also anti-tumoural effect, demonstrated *in vitro* and *in vivo* (27, 28). *In vitro,* authors demonstrated that the molecule was able to enhance cytoxicity of Natural Killer lymphocytes against HCC cells (29). Furthermore, IFN decreases proliferation and may induce apoptosis *in vitro* and *in vivo* (30-33), although these associations were not reproduced by other authors (34, 35).

Other drugs have been tested for tertiary prevention of HCC. Polyprenoic acid, a vitamin A derivative acyclic retinoid, has affinity for nuclear retinoid receptors, thereby inducing cell differentiation (36) and apoptosis (37). Polyprenoic acid prevented recurrence of a second HCC by nearly 50% after resection or percutaneous treatment in a small cohort of Japanese patients (38). No study was published that reproduced these impressive results and therefore the drug has not been implemented so far. Researchers from the same team have shown synergistic effects of acyclic retinoid and IFN-β *in vitro* (39) but the combination has not been tested in patients. More recently, other synergistic combinations using acyclic retinoid with trastuzumab, a monoclonal antibody blocking the *ErbB2* receptor (40), or acyclic retinoid and vitamin K2 (41) demonstrated anti-tumoural effect *in vitro.*

Immunotherapy could be another promising approach in the preventive setting. One RCT, using autologous stimulated lymphocytes injected in Japanese patients after curative resection showed an improvement in recurrence-free survival but not in overall survival (42). However, this treatment is technically demanding, needs expertise and is expensive.

Figure 3: temporal evolution of liver carcinogenesis



From (43)

C. The cirrhotic liver as a pre-neoplastic organ:

Numerous epidemiologic evidences exist to support cirrhosis as a condition associated with carcinogenesis, which develops after decades of chronic liver disease (Figure 3, (43)). First, it has been shown that HCC incidence increases in parallel to fibrosis stage (44), with a risk that remains low during chronic hepatitis stage, but rises exponentially at cirrhotic stage. Second, when recurrence of HCC occurs after a complete resection, this happens either early (within 2 years) and is considered as arising from occult intra-hepatic metastasis, or later, and is considered as *de novo* HCC. The risk factors and prognosis are different in these 2 categories of recurrence (45, 46). Very recently, Hoshida et al. published an important paper in the New England Journal of Medicine (47) that demonstrated quite convincingly that late recurrence of HCC was closely linked to gene expression profile from the cirrhotic background rather than the gene profile of the first resected tumour. These data further emphasize the assumption that cirrhosis is a preneoplastic organ

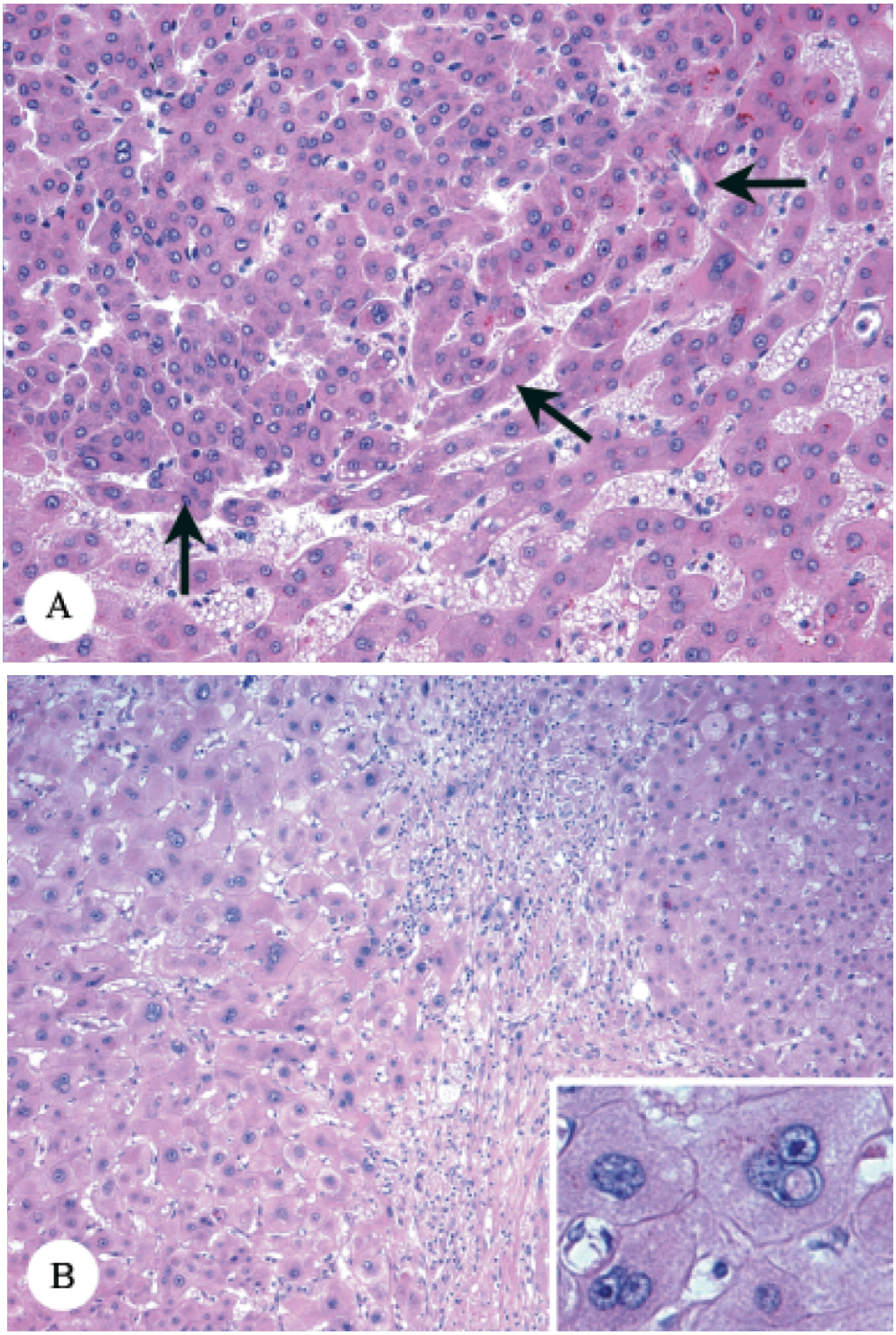
1. Pathologic progression:

From the histopathologic point-of-view, the sequence of normal liver to cancer comprises several steps. The first steps are characterized by the emergence of preneoplastic lesions (48). These comprise small- and large-cell dysplastic foci that can form nodules. Large cell dysplasia (LCD) is formed by cells with nuclear and cellular enlargement, normal nucleocytoplasmic ratio, gathering first in foci, sizing less than 1 mm (Fig.4B). LCD is also characterized by low proliferation, high apoptotic level and has morphologic molecular features of hepatocyte senescence (49, 50). These foci, also called “large cell change”, are thought to be bystander of advanced cirrhosis instead of real precursors of dysplastic nodules and HCC.

On the contrary, small cell dysplasia (also called “small cell change”) consists of cells with high nucleocytoplasmic ratio and smaller cytoplasmic compartment (Fig.4A). These cells are highly proliferative and show morphologic and molecular similarities with HCC (50, 51).

With time, foci can grow and form dysplastic nodules, which can be of low-grade (LGDN) or high-grade dysplasia (HGDN) (Fig.5). Morphological criteria for LGDN and HGDN are well established (52), but the molecular transition from dysplastic nodules to HCC is poorly understood. However, we know that loss of heterozygosity and chromosomal instability increases from cirrhosis to LGDN, from LGDN to HGDN, finally from HGDN to HCC (53-55). HGDN are thought to be precursors of HCC, with a 4-fold increased risk of developing HCC when HGDN are found at biopsy (56).

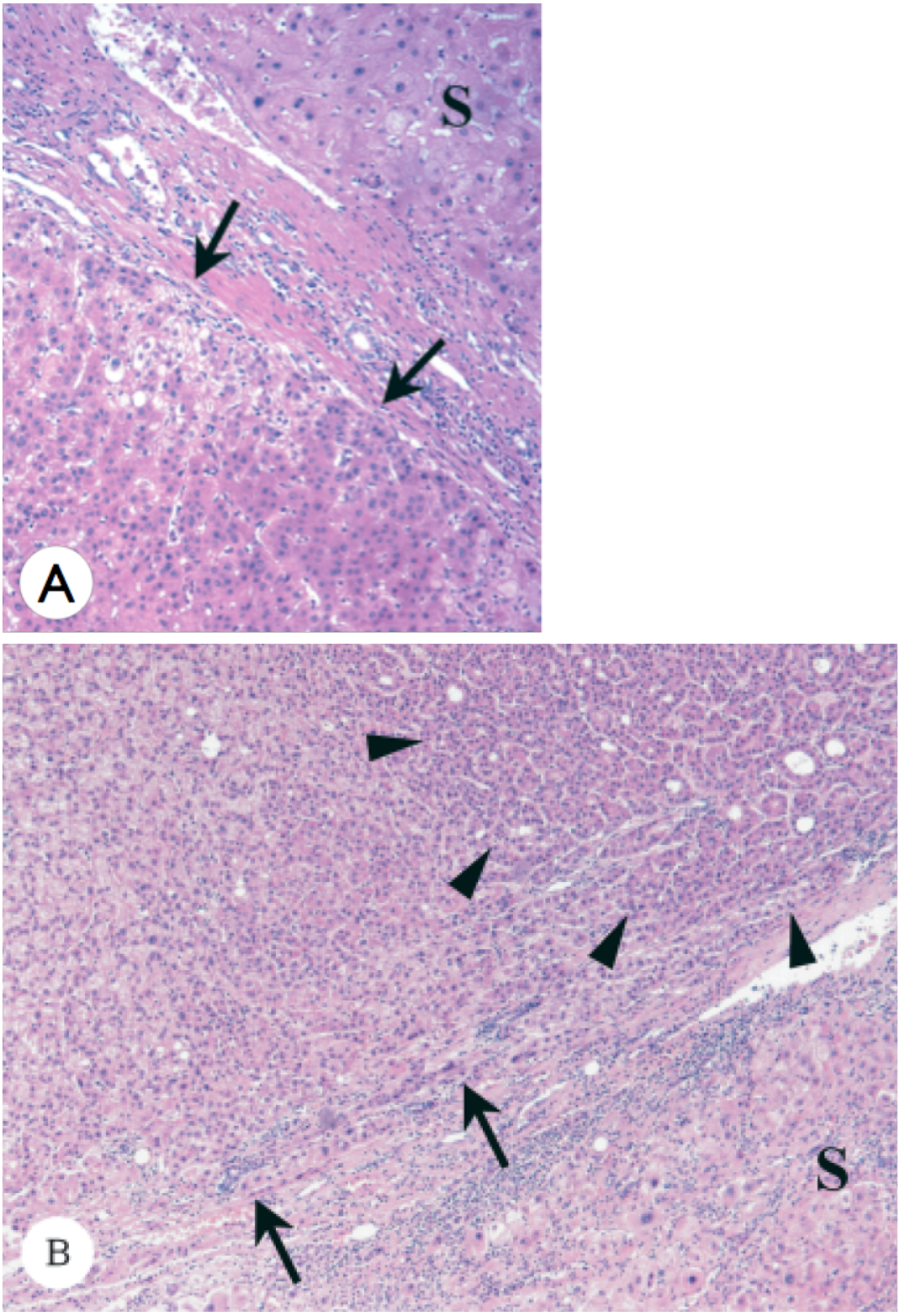
Figure 4, from (57):



A. Focus of small cell dysplasia, composed of small basophilic hepatocytes with a high nucleocytoplasmic ratio, depicted by arrows.

B. Large cell dysplasia, cells with nuclear and cellular enlargement, normal nucleocytoplasmic ratio, multinucleation and prominent nucleoli.

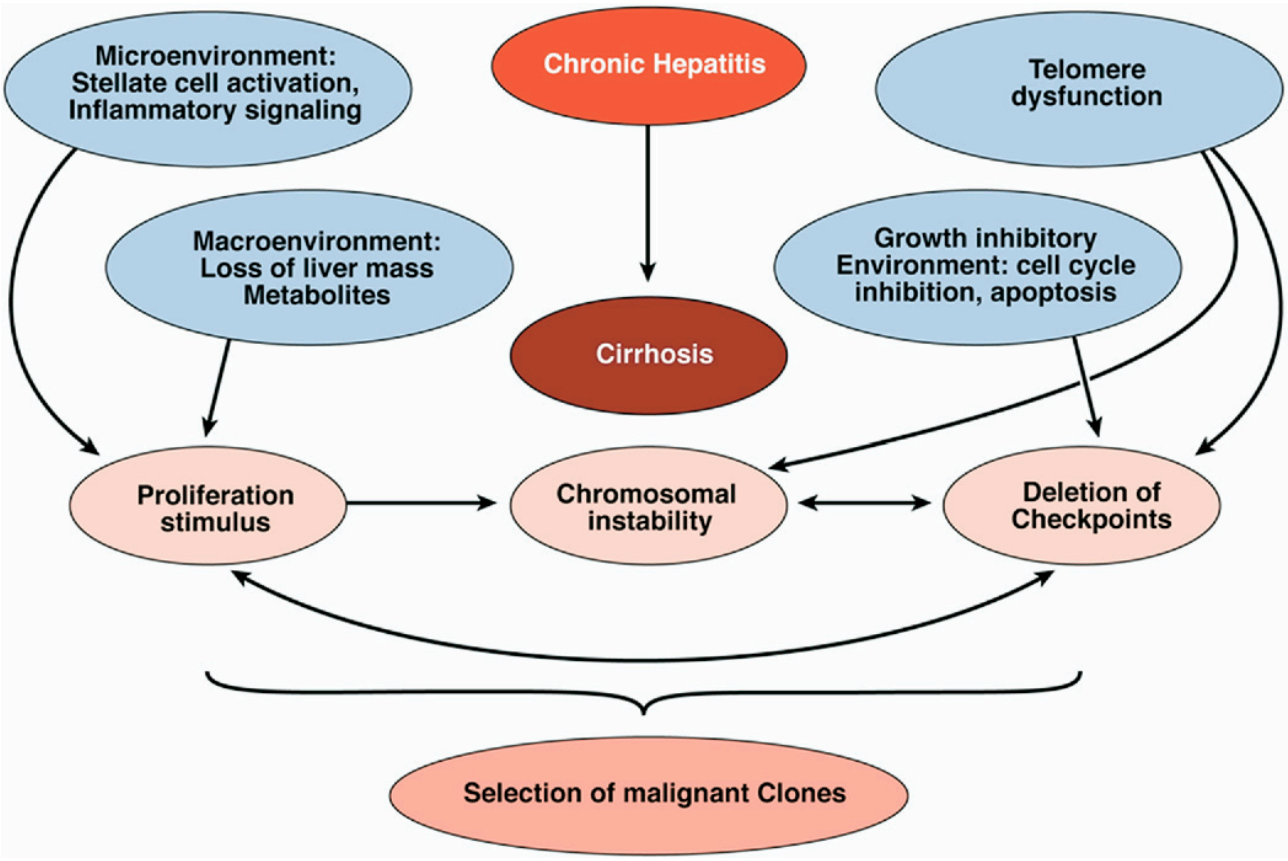
Figure 5; from (57):



A. Low grade dysplastic nodule, composed of small hepatocytes forming 2-3 cell thick trabecules. The cells are smaller than in the surrounding tissue (S), with a high nucleocytoplasmic ratio.

B. Inside a HGDN (arrows), a small HCC (arrowheads), formed of cells with increased cellular atypia, gathering in even thicker trabecules, showing also pseudoacini.

Figure 6:



From (1)

2. Molecular alterations during cirrhosis:

Liver carcinogenesis is highly complex, involves multiple molecular mechanisms and interactions with the milieu (Figure 6). These have been extensively reviewed in a recent publication (1). Some of them are worth revisiting because they will be of importance for the understanding of our research rationale and results.

a) Telomere shortening:

Telomeres are the special nucleoprotein structures that protect chromosome ends from both recombination and degradation. With aging, these structures shorten and limit the proliferative potential of all human cells to a finite number of divisions. During chronic liver disease, telomere shortening is accelerated, resulting in an accumulation of senescent cells at the cirrhotic stage (58). Once telomeres reach a critically short size, DNA damage signals are induced, together with apoptosis and cell cycle arrest. As short telomeres can resemble to DNA double strand breaks, the DNA repair machinery is also activated, that can lead to chromosomal fusion. These fusions lead to chromosome gain, loss or translocation during the cell cycle, thereby inducing chromosomal instability (59). It has been shown that during liver carcinogenesis, telomeres are gradually shortened, from cirrhosis to large cell change, then small cell change, finally in HCC (60), and that this shortening was associated with chromosomal instability and HCC development (61)

b) Impaired hepatocyte proliferation:

Independently of telomere shortening accompanying hepatocyte ageing, the decrease in hepatocyte proliferation itself could enhance tumour formation in the cirrhotic liver. This has thoroughly been studied using animal chemical models closely resembling the one we studied in our two first studies. van Gijssel et al have indeed shown that, using several chemical carcinogens, including DEN and AAF, they inhibited proliferation of normal hepatocytes in rodents via p53 overexpression, leading to cell cycle arrest, thereby giving a proliferative advantage to transformed cells that have a mutated, non functional p53 (62). p53, the “gate-keeper of the genome”, is a major tumour suppressor gene. The main target of p53 is p21, which induces cell cycle arrest and senescence in response to telomere shortening (63). Accordingly, authors have shown a correlation between increased p21 expression in cirrhosis and the risk of HCC development, suggesting its role as a bystander (64). In line with this hypothesis, others have demonstrated a high expression of the p21 protein in cirrhosis and large cell change, and much less expression in small cell change and HCC, together with much shorter telomeres, suggesting a continuum of the carcinogenic process, with progressive loss of telomeres, loss of the p53 cell cycle checkpoint function, and selective survival advantages for those cells that do not express these cell cycle gate-keepers (60). A decrease in liver regeneration could explain the acceleration of cirrhosis and maybe liver carcinogenesis in elderly patients suffering from chronic liver disease (65). Indeed, authors have shown, by implanting normal hepatocytes into livers of rats of various ages, that no implanted cells were found in young rats, in contrast to rats over 18 months, where a significant expansion of clonogenic clusters from donor-derived cells was observed (66).

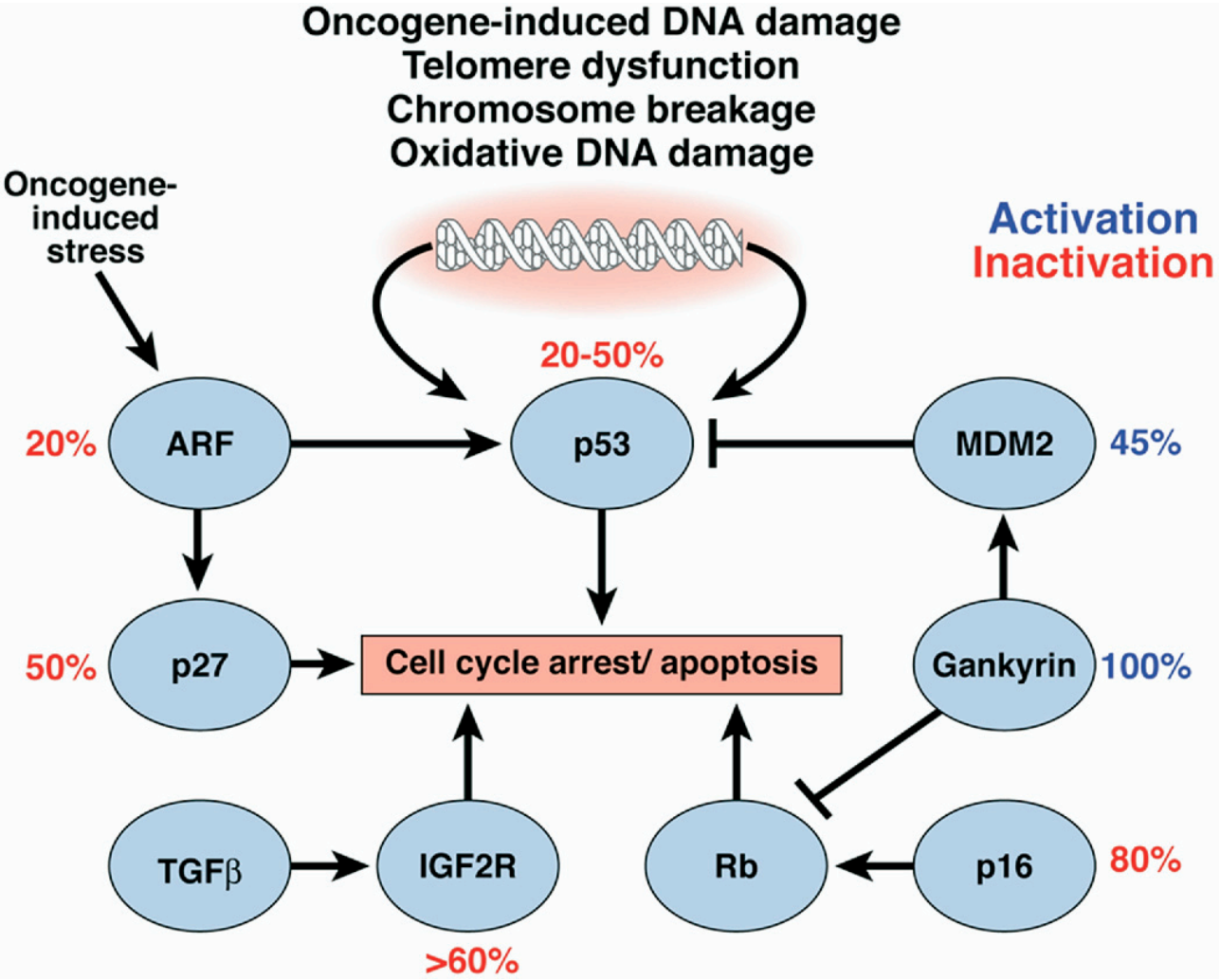
c) Interaction with the milieu: role of the micro- and macroenvironment.

The normal liver has the unique capacity to regenerate after partial resection, both in animal and humans. The regeneration is tightly regulated, and is thought to be due to an interaction between hepatic metabolites secreted after resection that will stimulate growth factors production, inducing liver growth. Precise mechanisms involved in this unique process are not well understood. In liver cirrhosis, the regenerative capacity is lost and could well be a strong stimulus to macro-environment growth factor production that in turn may well accelerate tumour formation, by selectively stimulating proliferation in transformed cells.

But cirrhosis also changes the microenvironment, and one of the hallmarks of these modifications implies stellate cell activation. Activated stellate cells produce extracellular matrix components, pro-inflammatory cytokines, growth factors and reactive oxygen species (67). Many of these components are known to be involved in tumour formation and invasion (68). Another important aspect of the microenvironment is the inflammatory response induced by the immune system in the context of chronic hepatitis. Along these lines, it has been shown that a unique immune response in the tissue surrounding the tumour is associated with venous encasement, recurrence and prognosis of HCC (69).

Nuclear factor κ B (NFκB) seems to have an important role in this interplay between HCC and the microenvironment. Authors have shown that when NFκB expression is inhibited only and specifically in hepatocytes in a mouse model of DEN-induced HCC, the inflammatory response, due to Kupffer cells production of pro-inflammatory and growth factors, was enhanced and with increased carcinogenesis (70). They also could show that the deletion of IKKβ, an activator of NFκB, not only in hepatocytes but also in Kupffer cells, inhibited hepatocarcinogenesis, thus

Figure 7, From (1):



concluding that NFκB activity in Kupffer cells contributes to cancer promotion and that NFκB activity in hepatocytes acts as a cancer suppressor (71).

3. Molecular alterations occurring in HCC:

a) Loss of cell cycle checkpoints:

Activation of cell cycle checkpoints impairs hepatocyte proliferation during cirrhosis (72). Abrogation of these checkpoints could confer a selective advantage allowing transformed cells to clonal expansion. In HCC, many checkpoints are abrogated, as shown in Figure 7.

Activation of the p53 pathway occurs after DNA damage or oncogene activation. Activation leads to cell cycle arrest and DNA repair and apoptosis. In HCC, p53 is frequently mutated and non functional in more than 60% of cases in aflatoxin B1-induced HCC (73) and in 20-40% of HCC not associated with aflatoxin B1 exposure. It has also been shown that Gankyrin, an inhibitor of both p53 and Retinoblastoma protein (Rb), is overexpressed in many HCCs, mainly at early stages of tumour development (74, 75). Gankyrin is a protein that binds to Murine double minute 2 (Mdm2), an oncogene involved in p53 ubiquitination leading to its degradation by the proteasome complex. Mdm2 itself is overexpressed in HCC (75, 76). Furthermore, inactivation of the tumor suppressor protein p14 alternate reading frame (ARF), which is also a regulator of p53, is found in 20% of HCC, mainly in the absence of p53 mutation (77). In addition to its effect on p53, ARF inactivation also leads to downregulation of p27, this downregulation being a negative prognostic factor in HCC (78)

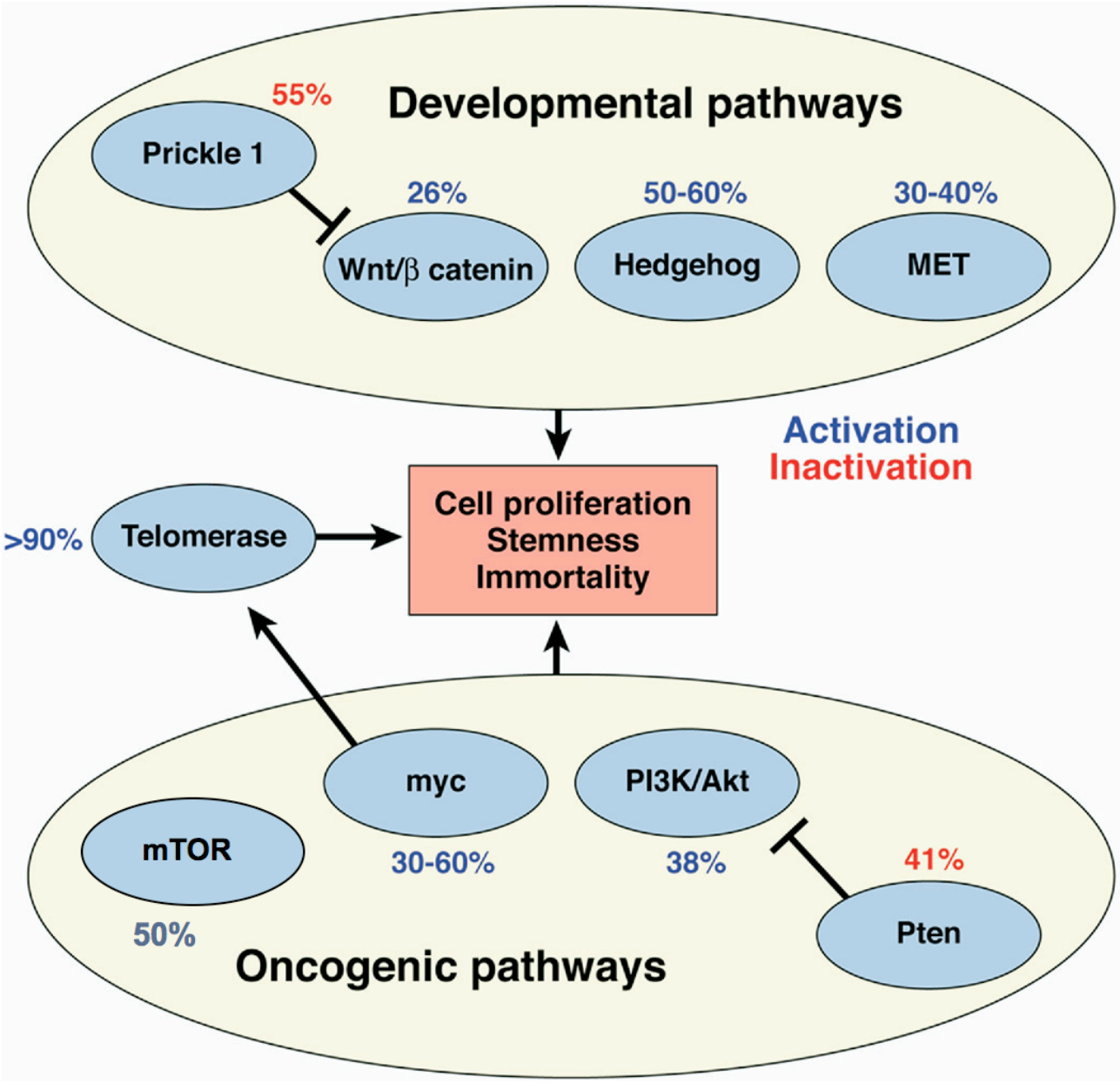
Another major pathway limiting cell proliferation in response to oncogene activation is the p16/Rb pathway, which is disrupted in about 80% of HCC (79). As previously mentioned, Gankyrin inhibits also the Rb pathway and is overexpressed in HCC and cirrhotic liver (75). It is thus conceivable that impairment of this cell cycle checkpoint would allow cell proliferation at the cirrhosis stage. In agreement with this assumption, it was found that p16 is overexpressed in cirrhosis and large cell change, but its expression was dramatically lower in small cell change and HCC (60). Furthermore, authors showed in Japanese cirrhotic patients that Inhibitor of differentiation/DNA binding protein 1 (Id-1, a inhibitor of the p16 protein) expression was found in 40% of cases (80). Id-1 expression was linked to an increased risk of HCC in that study.

Insulin-like growth factor 2 receptor (IGF2R) loss of heterozygosity is a frequent and early event in liver carcinogenesis (81, 82). IGF2R decreases cell proliferation by promoting degradation of the mitogen IGF2 and by activating the transforming growth factor β (TGFβ) pathway (83). IGF2 is overexpressed in HCC (84, 85) and, together with the impaired function of its receptor could represent a selective advantage at the cirrhosis stage by counteracting with TGFβ's pro-apoptotic and anti-proliferative actions.

b) Resistance to apoptosis:

P53 is frequently inactivated in HCC, as already mentioned. Resistance to p53-induced apoptosis can thus be one of the mechanisms promoting liver carcinogenesis. Accordingly, HCC overexpresses Hepatoma Subtracted-cDNA library Clone One (HSCO), an inhibitor of p53; in one study, HSCO suppressed p53-dependent apoptosis by preventing nuclear localisation of NFkB (86). Apoptosis can occur independently of p53 activation, via the TGFb pathway (87) and Akt pathway (88). Recently, authors have shown the role of a new apoptosis sensitizer, Hint2, acting at the mitochondrial level in

Figure 8:



Adapted from (1)

humans, and its low expression in HCC, linked to poor prognosis (89).

c) Activation of oncogenic pathways:

The main oncogenic pathways described in HCC are shown in Figure 8. They include so-called “developmental pathways” because they involve pathways involved in liver ontogeny, and “oncogenic pathways”. Among developmental pathways, the Wnt/beta-catenin pathway is frequently upregulated in rodent and human HCC (90). Activation of beta-catenin is sufficient to induce HCC in mouse models, by deletion of the *adenomatous polyposis coli* gene specifically in the hepatocytes (91). Authors have shown that the Prickle-1 protein suppressed activity of the Wnt/beta-catenin pathway *in vitro* and that Prickle-1 was underexpressed in 55% of human HCC (92). This low expression was linked to an accumulation of cellular beta-catenin and large tumour size. Other recently discovered pathways involved in liver carcinogenesis are the Hedgehog pathways, overexpressed in about 60% of HCC (93, 94), and the c-met proto-oncogene/hepatocyte growth factor pathway (95). *Myc* is a potent oncogene that induces HCC in mouse models, with tumours induced being very dependent on continuous activation of the oncogene (96). C-myc amplifications have been found in 38% of multifocal HCC, in 60% of metastatic HCC and less in early HCC (97). In line with this, increased copy number of mouse chromosome 15 (carrying the *myc* gene locus) is a frequent alteration in mouse HCC, in the setting of telomere shortening and p53 mutation (98). Another pathway, very complex in its understanding, but with promise in regard to therapy because of the development of very efficient inhibitors, is the mammalian target of rapamycin (mTOR) pathway. This pathway has been shown to be activated in many cancer types, including HCC (99, 100). Authors have shown anti-tumour activity of mTOR inhibitors in animal models (100-102) and clinical trials are ongoing (103). An activation of Akt signalling,

together with an impaired expression of its negative regulator phosphatase and tensin homolog (Pten), has been reported in 40-60% of HCC (104). Activation of Akt leads to the suppression of TGFβ-induced apoptosis (88).

The activation of several developmental pathways in HCC suggests the role of liver stem cells for some tumours to arise. Because of the growth inhibitory environment in cirrhosis, liver stem cells may be preferentially activated and transformed. Evidence exists concerning liver stem cells in rodents (105) and a stem cell gene expression signature has been identified in a subset of human HCC (106).

d) Tumour cell immortality:

The huge majority of HCC show up-regulated telomerase reverse transcriptase activity. Furthermore, telomerase is inactive in normal and cirrhotic liver, activation occurring during the transition from premalignant lesions to HCC (74). Experiments in rodents have demonstrated that, on the one hand, telomere shortening increases initiation of liver tumours, but on the other hand, telomerase deficiency limits their progression into macroscopic lesions (98, 107). A hypothesis for this interesting finding would be that the accumulation of chromosomal instability and DNA damage would activate the p53 cell cycle checkpoint pathway and its tumour suppressor activity, finally impairing tumour growth. Telomerase expression in this context would prevent DNA damage and p53 activation, thus inducing tumour progression. Authors have shown that telomerase expression could also prevent p53-independent tumour suppression (98).

In summary, cirrhosis and liver carcinogenesis are closely linked in regard with several aspects, recapitulated in Figure 6:

- telomere dysfunction leading to chromosomal instability, and dysfunction of several cell cycle checkpoints.
- a growth-inhibitory environment, giving a selective advantage to transformed proliferative cells.
- Proliferative stimuli by the macro- and microenvironment.

D. Animal models of HCC:

As mentioned previously, HCC arises in an inflammatory environment, driving proliferation through growth factor production and stimulating senescent cells that escape cell cycle checkpoints due to chromosomal instability. Reproducing such events in animal models is difficult, so that no perfect animal model exists. In line with the many aetiologies, and various pathways involved, a multitude of models have been evaluated. A thorough review of animal models of HCC has recently been published (108). These can be subdivided into: xenograft models, viral models, models recapitulating molecular events (cell cycling, telomere dysfunction, growth factor signalling, developmental and oncogenic pathways), models of chemically-induced fibrosis and carcinogenesis; finally genetically engineered models for fibrosis and HCC. Each of these models approaches one of the characteristics of HCC, very few integrate more than one, e.g. fibrosis and telomere shortening (98). To evaluate possible similarities between rodent and human carcinogenesis, one very interesting approach, studied by Thorgeirsson and colleagues, is the comparison of gene expression patterns in transgenic mouse models and human HCC (109). In their studies, the authors could show expression profiles similar to patients with good prognosis in transgenic mice overexpressing myc, E2f1 and Myc/E2f1; and similar to HCC patients with poor survival in transgenic mice with overexpression of Myc/TgFα and, interestingly for our study, also in animals treated with the carcinogen DEN. This study supports the conclusion that comparing gene expression profiles of the two species by applying genome wide microarray analysis could help identifying the best-fit mouse models.

In order to understand liver carcinogenesis, several chemical agents have been used for induction of pre-neoplastic and neoplastic foci, nodules and tumours in animals. Sequential administrations of carcinogens allow the study of very early

carcinogenic events, thought to be similar between rodents and humans. It was proposed, in line with a general assumption, that also in the liver, the first event in carcinogenesis would be an “initiation phase”, caused by a mutagen that would result in irreversible mutations. *Per se*, these mutations would of course not be sufficient to give rise to neoplasms, but in the liver of rodents, would be associated with the appearance of so-called “foci of altered hepatocytes” (FAH). To grow, these foci need to be boosted by a promoting agent. The “promotion phase” is not associated with induction of mutations and is reversible upon arrest of the stimulus. During this phase, most mutated cells undergo apoptosis but a few respond to promoting agents presumably due to a selective advantage and evolve to dysplastic foci, then nodules. If the promoting stimulus is removed, these foci will disappear, undergoing massive apoptosis (110). Finally, a third step, called “progression phase” is driven by a mutagen, causing irreversible DNA changes sufficient for neoplasms to appear and maintain.

The first model ever described in rats was the Solt-Farber model (111) where DEN was used as a stimulus for initiation, then AAF, as a promoter, to give a selective pressure to mutated cells with a survival and proliferative advantage in the face of a strong proliferating stimulus, i.e. partial hepatectomy. AAF has been shown to give a selective advantage to initiated hepatocytes, the mechanism being a mito-inhibitory effect on those phenotypically normal cells, through up-regulation of p53 and its effector p21, which are not expressed in FAH (112).

Subsequently, many chemicals were used, in a combined or sequential fashion (e.g; DEN+AAF; DEN+phenobarbital, DEN+thioacetamide..), with various phenotypic changes induced, either preneoplastic or neoplasic. Mechanisms of action are either pure genotoxic effects (e.g. DEN, AAF, DMN) or oxidative stress induction (thioacetamide), or other (phenobarbital).

DEN is a DNA alkylating agent, rapidly metabolized to reactive metabolites that interact with DNA to form adducts leading to genetic alterations (113). In rats, depending on the dose and mode of administration, it can either give rise rapidly to cirrhosis without evidence of HCC (114), HCC without cirrhosis (115), or cirrhosis and HCC in 16-18 weeks (116). This latter model is very interesting because of the coexistence of the cirrhotic microenvironment with HCC. Authors also showed, as in human HCC, an overexpression of the Epidermal Growth Factor Receptor (EGFR) ligand transforming growth factor α (TGFα) in these DEN-induced HCCs (117). In another animal model using DEN in rats, authors have shown beta-catenin mutations in 45% of HCC (118). As already mentioned, when used in mice, DEN induces HCC wich express a panel of genes also found in human HCC with a poor prognosis (109).

The administration of DEN initiates the appearance of cells that show a different phenotype and the expression of specific proteins that can be highlighted by immunohistochemistry. The protein that has been shown to be specifically overexpressed in FAH is the « placental form of Glutathione S-transferase » of the rat, called GSTp or GST 7-7 (119). This enzyme belongs to the phase II detoxification enzyme family but, unlike other GSTs, is not induced by xenobiotics. GSTp is consistently and rapidly expressed in various models of preneoplasia but not in non genotoxic models and poorly during liver regeneration (120). Its expression is regulated by an enhancer element, GST-p enhancer 1 (GPE1) (121). In the normal liver, GPE1 binds with CAAT enhancer binding protein alpha (C/EBPα), thereby silencing the GSTp gene (122). C/EBPα is highly expressed in the liver, and functions to maintain liver quiescence through its antiproliferative activity (123). Reduced expression of C/EBPα has been found in HCC (124). After administration of a carcinogen, C/EBPα expression is down regulated and substituted for by the heterodimer Nrf2/MafK in the pre-neoplastic cells (125). This substitution leads to transcriptional activation of the GSTp gene, through histone

acetylation. So, while these recent works show that the expression of the GSTp gene is co-regulated by genes associated with cellular proliferation and transformation, i.e, C/EBPα and Nrf2/MafK, relation between changes in GSTp expression and the first molecular events of liver carcinogenesis remains to be elucidated. GSTp positive cells and foci can be easily quantified both in size and number using morphometric methods (126, 127), allowing precise analysis of the effect of various carcinogens, but also of the effect of chemopreventive drugs.

Our work has focused on two different steps in the chronology of liver carcinogenesis. First, we used a sequential two-stage model using DEN and AAF, to analyse the effect of our drugs in the very early events of carcinogenesis, i.e. cellular initiation and promotion that lead to preneoplasia. Because these molecular events have been well characterized in that model and because of similarities of these events with human carcinogenesis, we believe the model confers to our findings some validity in regard with the clinical setting. Second, we wanted to use a model that provided the inflammatory and fibrotic micro-environment and that could give rise to established dysplastic, neoplastic nodules and HCC. Therefore the model using repeated injections of DEN only was chosen, because this model gives rise to significant fibrosis in 12 weeks, and coexisting cirrhosis and HCC after 16 weeks. Using this model, we aimed at assessing the effect of the medications at a more advanced stage, where cells are irreversibly engaged in carcinogenesis.

E. Chemoprevention in animal models: what has already been done?

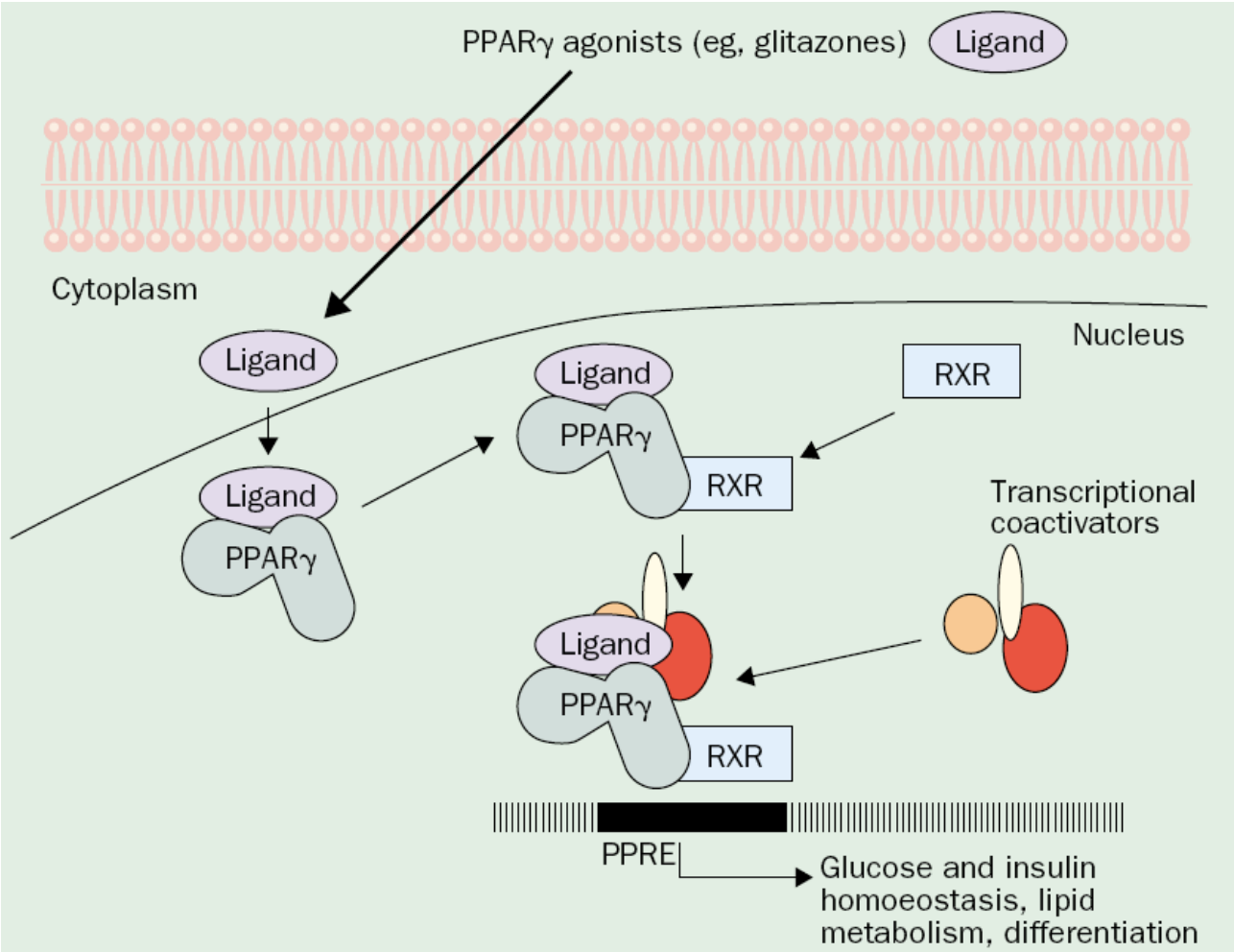
Many experimental studies have evaluated the role of chemopreventive agents in animal models of HCC. Authors have used models of chemically induced neoplastic lesions, either in the setting of fibrosis, using DEN (116) or not, using DEN and AAF (33); transgenic mice (128) or dietary models, e.g. a choline deficient ethionine-supplemented diet in rats (129). Many different drugs have been studied, including antivirals (33, 130), traditional medicinal herbs (128, 131) or targeted therapies like gefitinib, a tyrosine kinase inhibitor of EGFR (116), imatinib mesylate, a tyrosine kinase inhibitor of the c-kit receptor (129) or MEK inhibitors (132). Also, different aetiogenic pathways have been addressed, such as inflammation, oxidative stress, oncogenic pathways. Among those, agents targeting oxidative stress and chronic inflammation associated with cirrhosis are particularly relevant to this work and will be highlighted in this chapter.

The role of the micro-environment is of importance in liver carcinogenesis, as recently demonstrated by authors in the New England Journal of Medicine (47). Therefore the work performed by Grisham and Thorgeirsson is worth commenting. Already ten years ago, these authors developed a mouse model of HCC, using the overexpressed transgenes c-myc and TGFα (133). The particularity of this model is that it promotes overproduction of reactive oxygen species (ROS) and thus creates an oxidative stress environment. They could later demonstrate that this model gives rise to HCC with a molecular signature similar to that of human HCC with a poor prognosis (109), as the DEN model we used. ROS are known to induce oxidative DNA damage and promote cancer (134). Based on that, they demonstrated a chemopreventive effect of the anti-oxidant compound vitamin E, by decreasing the number of liver adenomas and preventing malignant transformation in TGFα/ c-myc transgenic mice (135). This founder work was

reproduced with other anti-oxidant, like curcumin (136), or selenium compounds (137, 138) in different animal models and by independent teams, which strengthens the proof of concept of a chemopreventive approach with anti-oxidant compounds.

Anti-inflammatory compounds have also been analysed, such as aspirin, piroxicam and COX-2 inhibitors. Several lines of evidence suggest that prostaglandins are involved in liver carcinogenesis. These have been reviewed recently (139). In a preneoplastic *in vivo* model of foci of altered hepatocytes (FAH) induced by a choline-deficient diet, authors have demonstrated an increased expression of COX-2 in FAH and the chemopreventive effect of nimesulide, a COX-2 inhibitor (140). Others have demonstrated the efficacy of celecoxib, another COX-2 inhibitor, in preventing FAH induced by the Solt-Farber model (141). More recently, the efficacy of the COX-2 inhibitor etodolac has been studied in fatty liver Shionogi mice, animals that develop severe steatosis and spontaneous HCC (142). Interestingly, authors showed a decrease in prostaglandin E2, but also a diminished RXRα expression in non-tumoural tissue in etodolac-treated animals, suggesting a combined effect on both the RXR and anti-inflammatory pathways.

Figure 9:



from (144)

F. Peroxisome proliferator-activating receptors gamma (PPARγ) and agonists:

PPARγ is a nuclear transcription factor, member of the nuclear hormone receptor superfamily, being activated by binding its ligand, then heterodimerizing with RXR, to finally bind with specific response elements in the nucleus, called peroxisome proliferating response elements (PPRE) (Figure 9). Activation of PPARγ by agonists such as thiazolidinediones (TZDs) has been shown to have anti-cancer effect *in vitro* and *in vivo* in many cancer types (143, 144). However, in some situations, like in colon cancer models, PPARγ agonists can have tumour promoting effects (145). Effects of PPARγ agonists on HCC have been studied in the last years both in cell cultures and in animal models. We have recently reviewed the role of PPARγ in hepatocellular carcinoma (146). Here we summarize the main findings of these works and attempt to define the role of PPARγ and its modulation in HCC.

1. Expression of PPARγ in rodent and human liver:

PPARγ RNA and protein expression has been demonstrated *in vitro* in several cell lines, including Hep3B, HepG2, Huh7 and others (147-152). Its expression was variable among cell lines, with mRNA levels not necessarily correlating protein expression.

In human HCC, reports from 3 papers show conflicting results: in the first paper, PPARγ protein expression was assessed by western-blot (WB) in 5 cirrhotic patients and showed no difference between HCC and non-tumoral cirrhotic liver (153). The second paper showed a constant overexpression of PPARγ protein in 20 HCCs and no expression

in surrounding liver, assessed by immunohistochemistry (IHC). In this paper, no information was given regarding the presence of cirrhosis (154). Finally, a recent report, analysing mRNA and protein expression (by WB) in 20 patients, with cirrhosis (n=12) and chronic hepatitis (n=8), showed a statistically significant decrease in PPARγ expression in HCC compared to adjacent liver (155). Furthermore, PPARγ mRNA was less expressed in poorly differentiated HCC compared to well differentiated ones. The last 2 results, using 2 different methods (IHC vs WB) in different populations, can therefore not be compared, and certainly other analyses will be needed before we can draw any conclusions on PPARγ expression in HCC and cirrhosis compared to normal liver, where it is known to be low (156).

2. Effect of PPARγ agonists on cell proliferation

The effect of the natural PPARγ agonist 15-deoxy-Δ12, 14-prostaglandin J2 and the synthetic agonists thiazolidinediones has been tested in many human HCC cell cultures (147, 148, 150, 152, 153, 155, 157, 158). Overall, cell growth was inhibited, at variable concentrations. Troglitazone, the most studied TZD, was shown to inhibit cell growth already at 5-10 mMol concentrations, although some contradictory reports have been published (151). Pioglitazone and ciglitazone, the other main TZDs tested, were less active, with concentrations from 50 to 100 mMol needed to have significant growth inhibitory effect. The anti-proliferative effect was shown to be due to a cell cycle arrest, with cells accumulating at the G1 phase. Koga et al have performed the most thorough mechanistic investigations in the field (148, 153). Using Troglitazone at a dose of 50 mMol, they could show on several cell lines that the inhibitory effect on cell cycle was linked to an increase in the mRNA and protein expression of the Cyclin dependent kinase inhibitor (Cki) $p21^{waf1/cip1}$ and also an increase in protein expression only of $p27^{kip1}$, another CKi. These proteins were shown to bind to the Cyclin dependent kinase CDK2, thus inhibiting

its activation. Because the intracellular concentration of $p27^{kip1}$ is tightly regulated by its degradation through the ubiquitin-proteasome pathway, the authors have analysed and could show that indeed, troglitazone had an inhibitory effect on $p27^{kip1}$ degradation via inhibition of Skp2, an F-box protein of ubiquitin-ligase complex. These results were reproduced by others (152, 155, 159).

In vivo also, authors could show an inhibition of the development of pre-neoplastic and neoplastic liver nodules by PPARγ agonists. Kawaguchi et al (160), using pioglitazone in male Wistar rats fed with a choline-deficient L-amino acid defined diet, which is an animal model of non-alcoholic steatohepatitis model, showed that pioglitazone, at a concentration of 0.01% *wt/wt*, inhibited the formation of GSTp positive foci by a factor 2. Guo et al (161), using three TZDs, namely troglitazone, rosiglitazone and pioglitazone, in male Wistar rats showed that all could suppress the formation of GSTp foci that were induced by the strong mutagen diethylnitrosamine (DEN). In a longer experiment aiming at tumor formation, they found that pioglitazone at a 200 ppm concentration, equivalent to 0.05% *wt/wt*, prevented the appearance of macroscopic tumors. However, in this paper, no histologic proof of hepatocellular carcinoma was demonstrated, and the quantitation of tumors is subject to criticism, with a “stereomicroscopical” method used that is not described. Finally, Yu et al published their results on the effect of troglitazone, *in vitro* and *in vivo* in a nude mice model, injected subcutaneously with the human Huh7 HCC cell line (155). They could show quite convincingly that troglitazone, at the 200 ppm concentration, either inhibited the appearance of HCC, or decreased the growth of existing ones. The authors demonstrated that troglitazone induced a decrease in proliferation, assessed by PCNA, an increase in $p27^{kip1}$ and a decrease of COX-2 expression in tumors compared to controls.

It is important to mention that none of these studies using TZDs, has clearly demonstrated that the anti-proliferative effect of these drugs is due to activation of PPARγ pathway. In other cancer types, authors have demonstrated that troglitazone and

ciglitazone could inhibit cell proliferation in PPARγ $^{-/-}$ cell lines, by inhibition of translation initiation (162).

3. Role of PPARγ in the progression of liver fibrosis

An important paper published in 2002 by Galli et al (163) demonstrated that, given early, TZDs significantly reduced the development of liver fibrosis induced in rats, either by chronic administration of dimethylnitrosamine, carbon tetrachloride, or bile duct ligation. This anti-fibrotic effect was driven through a PPARγ dependant decreased activation of hepatic stellate cells. These early results showing the importance of PPARγ activation in the pathogenesis of fibrosis were confirmed by others (164, 165). Though, data from our team showed that the protective effect was very dependant on the rodent type (166) and the severity of fibrosis at the time of initiation of treatment (167). These data are thus to be interpreted with caution before going to clinical application. In humans, one randomised double-blind placebo-controlled study assessed the effect of pioglitazone vs. placebo in patients with NASH (168). One of the aims of the study was to assess fibrosis on liver biopsy before and after 6 months of a calorie-restricted diet with or without pioglitazone. The study failed to show differences in terms of fibrosis regression, assessed by histology, between groups (p=0.08), although patients in the pioglitazone group showed significantly less fibrosis after the 6 month regimen compared to before treatment (p=0.002).

4. PPARγ modulation in association with cell death

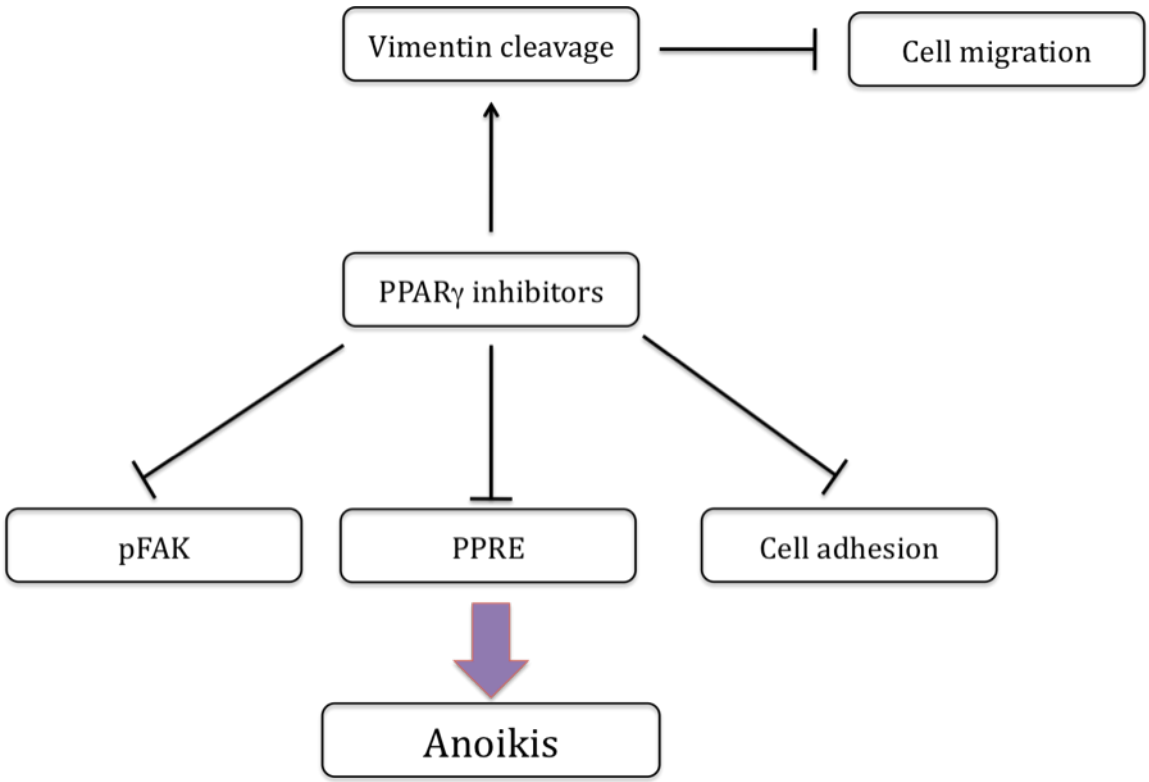
The role of PPARγ in apoptosis and anoikis (a specific type of apoptosis induced by loss of cell adhesion or inappropriate cell adhesion) is less clear. Several *in vitro* works report induction of apoptosis in HCC cell lines, constantly by troglitazone and not by others

TZDs (150, 155, 169-171). The main apoptotic mechanisms remain poorly understood, although some publications report an effect of troglitazone on the bax/bcl-2 balance (171), suggesting a role for the mitochondrial pathway. In another work, authors have analysed the effect of the natural PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (15D-JG2) on apoptosis of SK-Hep1 and hepG2 cells. They indeed showed that given at a dose of 50 mMol, 15D-JG2 could induce apoptosis by caspase 3 activation. Using PPARγ antisense oligodeoxynucleotides, they demonstrated that apoptosis was induced independently of PPARγ expression. They also showed that 15d-PGJ2 inhibited NF-kB activation induced by TNFα when PPARγ was normally expressed or down-regulated.

5. Is there a role for PPARγ inhibition in cancer?

Although, as discussed earlier in the text, the vast majority of available data deals with PPARγ agonists, some authors analyzed the effect of PPARγ inhibitors on cancer cell growth. One publication reported the effect of PPARγ inhibitors on cell adhesion and anoikis (154). On the contrary to the paper by Yu et al (155), authors found overexpression of PPARγ in human HCC. They showed that PPARγ inhibitors increased loss of adherence *in vitro* followed by caspase-dependent apoptosis, a finding reproduced using PPARγ siRNAs, reflecting the specificity of the PPARγ-dependent activation driving cell death. This was linked with a maintained B1-integrin expression (a protein involved in cell binding with extracellular matrix) but a decreased phosphorylation of FAK, a protein involved in cell adhesion downstream to integrins. They also showed that inhibitors were at least 5-fold more potent in reducing cell PPARγ than troglitazone and rosiglitazone. Another study evaluated the effect of PPARγ agonists and antagonists on cell growth,

Figure 10: Putative effects of PPARγ inhibitors



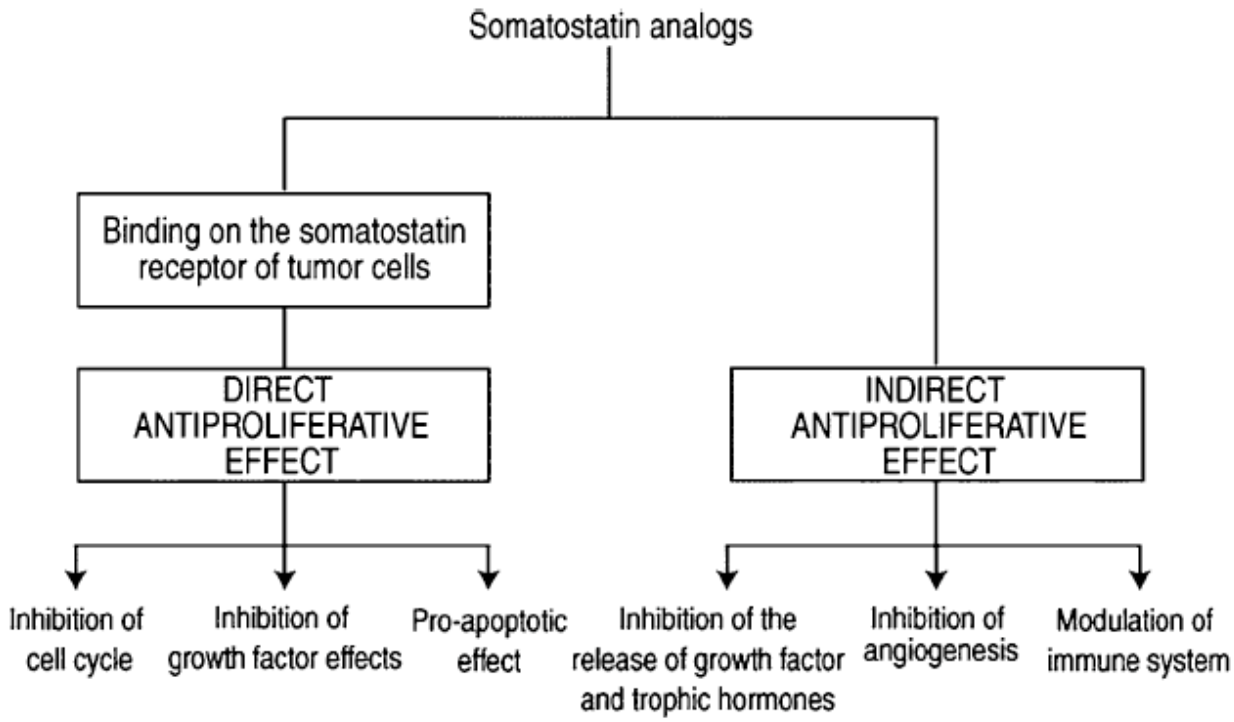
migration and invasion in four different HCC cell lines (172). Antagonists inhibited cell growth and migration more efficiently than PPARγ agonists. It was suggested that this effect could be due to Vimentin cleavage, thus interacting negatively with the cellular cytoskeleton. The putative effects of PPARγ inhibitors are illustrated in Figure 10.

Thus, several in vitro and in vivo papers suggest a role of the PPARγ pathway in the prevention and treatment of HCC. PPARγ mRNA and protein expression is constantly found in cell lines. In normal liver, it is known to be expressed at low levels, ten times less than in adipose tissue. We do not know its actual level of expression, neither in cirrhotic non-tumoral liver, nor in HCC, with conflicting results reported. Thiazolidinediones, which are active and specific PPARγ agonists, have shown anti-tumoral activity in cell lines and in animal models. This anticancer activity is due to inhibition of cell proliferation, by interfering with important cell cycle cyclins and cyclin-dependent kinase inhibitors, such as $p27^{kip1}$, but also by a pro-apoptotic action. The link between activation of the PPARγ pathway and the anticancer activity of TZDs is, however, not established. Finally, PPARγ antagonists have shown anti-tumor activity, by different mechanisms, mainly involving loss of cell adherence, migration and invasion.

G. Somatostatin analogues:

The second class of drugs that has a rationale for chemopreventive use in HCC is the class of somatostatin analogues. Somatostatins (SMS) are natural hormones, produced by the endocrine cells of the gastrointestinal tract, but also by neuronal cells, as well as some tumours (173). There exist 2 forms of SMS, namely SMS-14 and SMS-28, and both have many biological effects throughout the body. These effects are mainly inhibitory on the secretion of hormones, but also anti-proliferative, both on normal and tumour cells (174). However, due to their very short half-life (a few minutes), their use as anticancer and endocrine secretion inhibitors is limited in practice. Synthetic analogues have therefore been developed, with longer half-lives. Two are currently used in clinical practice: octreotide and lanreotide. These analogues are shorter peptides than SMS and lack the key enzyme cleavage site, providing stability. Their half-lives are approximately 110 min for octreotide (175) and 4.52 days for lanreotide (176). Formulations with much longer half-lives have been developed, such prolonged release lanreotide (25.5 days) (177) and even longer with long acting octreotide. SMS and analogues have biological effects through binding with membranous somatostatin receptors (sstr). Five sstr have been identified (sstr1-5), SMS binding with high affinity to all five, while octreotide and lanreotide have high affinity to sstr2, moderate similar affinity to sstr3 and sstr5, and low affinity to sstr1 and sstr4 (173, 178). In the clinical setting, both octreotide and lanreotide are inhibiting Growth Hormone (GH) and Insulin growth factor 1 (IGF-1) and are used to treat GH producing tumours (179) and digestive neuro-endocrine tumours (DNET). The anti-tumoural activity of analogues in DNET resides on the presence in generally great abundance of sstr on the cancer cell surface, sstr2 being the most frequently expressed subtype (180). By binding to sstr, they exert anti-tumoural effect, which is prominent in GH-producing tumours (181) and consists mainly in tumour stabilization in DNET (182).

Figure 11:



from (185)

However, because some tumours (the Swarm chondrosarcomas) are inhibited by SMS analogues despite no sstr expression (183, 184), the hypothesis of direct, i.e. dependent upon sstr binding, and indirect anticancer effect was proposed (185) and is depicted in Figure 11.

Direct effects are mediated through sstr binding. SMS and SMS analogues have shown direct anti-tumoural activity in many cancer cell types *in vitro* and *in vivo*, including prostate, lung, mammary, gastric, colo-rectal and pancreatic tumours, many of these tumours expressing sstr2 (186-188). Following binding with sstr, SMS analogues exert anticancer activity by inhibiting proliferation or inducing apoptosis, by initiating specific signal transduction pathways. So, each receptor subtype induces different pathways and has specific biological activity (189). Receptor subtypes involved in growth arrest are sstr 1, 2, 3 and 5. Sstr4 can have both inhibitory and stimulatory effect on proliferation, depending on the cell type. Also, the same pathway, e.g. the Mitogen Activated Protein Kinase/extracellular signal-regulated (MAPK/ERK) pathway can lead to either proliferation, via sstr4, or growth arrest, via sstr1 and/or sstr2, in different cell types (190, 191). Apoptosis induction may be mediated through the sstr3 subtype, by up-regulation of p53 and induction of the pro-apoptotic BAX protein, induced by octreotide (192).

Indirect antitumour effects of SMS and its analogues consist mainly of inhibition of the release of growth factors; antiangiogenic effects that will reduce blood flow to the tumour; and immunomodulatory effects that will stimulate the body's natural anticancer effect. Hormones like insulin, gastrin, IGF-1, Endothelial growth factor (EGF), TGFα and GH have promoting effects on tumours. Inhibition of IGF-1 secretion by the pituitary gland is a well-known effect of SMS analogues. They can act centrally by inhibiting GH synthesis, through binding with sstr 2 and sstr 5, thus suppressing the GH-IGF-1 axis. They can also act peripherally by inhibiting hepatic growth factor-induced

IGF-1 secretion, via sstr 2 and sstr 3 mediated dephosphorylation of STAT5b in rat hepatocytes (193). Angiogenesis, the mechanism by which tumours induce blood vessel formation from existing capillaries, is a very important feature of carcinogenesis (194). Many tumoural vessels express sstr, with high affinity for SMS analogues, independently of receptor expression in the tumour (195). Reubi et al have also shown that normal veins express sstr2 with high density (180) and others have found that sstr2 was overexpressed in proliferating endothelial cells, during the angiogenic switch (195). Sstr3 and 5 could also be involved in angiogenesis. The main mechanism by which SMS and analogues inhibit angiogenesis seems to be by a decrease in vascular endothelial growth factor (VEGF) synthesis (196), but also by affecting both the release and activity of other growth factors, such as platelet-derived growth factor (PDGF), IGF-1 and basic fibroblast growth factor (197).

On the contrary to normal liver, where no significant expression of sstr was found (198), in human HCC, sstr are expressed with inter-tumoural heterogeneity (199, 200). In the most thorough analysis of each sstr protein expression in HCC, Blaker et al have found that at least one sstr was expressed in 82% of the 56 HCC analyzed (200). Looking at each subtype, they found sstr5 to be expressed in 75% of HCC, then sstr3 in 64%, sstr 1 in 46%, sstr 2 in 41%. A minority of tumours expressed several sstr (e.g. sstr 1, 3 and 5 in 18%), while sstr 4 expression is not expressed in HCC. Some authors have demonstrated direct anti-tumour effect *in vitro* in HCC cell lines, using octreotide (201) or lanreotide (202). Furthermore, octreotide has shown indirect anticancer activity, by inhibiting angiogenesis in a HCC xenograft model (196). However, several randomized clinical trials testing SMS analogues have shown controversial results, both positive (203, 204) and negative (205-207). Authors, analysing the expression of sstr 2 using 111-Indium labelled octreotide scintigraphy, have also found contradictory results, some showing a negative impact on survival of sstr 2 expression, while others not (204, 208).

Because of the potential activity of SMS analogues on relevant pathways in liver carcinogenesis (i.e. cell cycle arrest, induction of apoptosis, inhibition of angiogenesis), we have used lanreotide in a preventive setting in animal models, an approach that has not been evaluated by others.

II. Aims of the study:

- To analyse the effect of pioglitazone, an agonist of PPARγ, on the inhibition of early carcinogenesis in the rat liver. For this we used a chemical model that induces foci of altered hepatocytes (FAH), which represent the first step in cellular transformation towards neoplasia. These results were published in the *European Journal of Cancer* in June 2007, and are shown in chapter III.
- In the same model of induction of pre-neoplastic foci, we assessed the effect of lanreotide, a somatostatin analogue. These experiments led to a publication in *Cancer Science* in December 2007, and are described in chapter IV.
- Finally, we analysed the effects of both pioglitazone and lanreotide in a very different setting, using a model that induces HCC in a cirrhotic environment. We tested the efficacy of the drugs when they were given at the start of the induction of fibrosis and HCC, or later on, when significant fibrosis was present. These results are shown in chapter V and VI, and are submitted for publication.

III. The PPARgamma agonist pioglitazone inhibits early neoplastic occurrence in the rat liver

In this study, using quantitative morphometry and Western blot, we showed that pioglitazone significantly decreases the size of preneoplastic foci. Analysis of proliferation and apoptosis, assessed by immunohistochemistry, demonstrated decreased proliferation but no effect on cell death in rats treated with pioglitazone. These events were associated with an increased expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$, compared to the non-treated group.

IV. Inhibition of early preneoplastic events in the rat liver by the somatostatin analog lanreotide

In this sudy, as well as for PGZ, we could show a decrease of FAH induced by DEN and AAF. We demonstrated an anti-proliferative but also a pro-apoptotic effect of LAN on preneoplastic hepatocytes. The mechanisms thought to underlie these effects are also increased expression levels of both p21 and p27, linked to a profound inhibition of Cyclin D1, an important actor of the cell cycle, responsible for the G1-S transition phase.

V. Efficacy of lanreotide in preventing the occurrence of chemically induced hepatocellular carcinoma in rats.

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Article Submitted

Abstract:

Hepatocellular carcinoma (HCC) is increasing worldwide and is the third cause of cancer-related death. HCC develops in a preneoplastic organ, the cirrhotic liver. Therefore, chemoprevention could play a role in the therapy of HCC. We have previously shown that lanreotide, a somatostatin analogue, inhibits the development of so-called "foci of altered hepatocytes", which represent very early neoplastic changes in rat liver. Here we induced *bona fide* HCC, by a different chemically induced model, known to lead to significant fibrosis before HCC appearance. Lanreotide was given at 2 different times: from the beginning of the experiment in one group and at the time when significant fibrosis was present in the second group. We showed that lanreotide decreased the number of HCC in both groups. We could demonstrate a significant decrease of hepatocyte proliferation and inhibition of fibrosis in the 2 groups. When given at the start of the experiment, lanreotide dramatically decreased angiogenic factors and enhanced apoptosis. This anti-tumoral effect of lanreotide deserves further work to assess the mechanistic relationship of its anti-proliferative and anti-angiogenic actions on liver neoplastic cells.

Introduction:

Hepatocellular carcinoma (HCC) represents a major health problem. Worldwide, it accounts for more than 500.000 deaths yearly (209). In western countries and Japan, the tumour develops in patients with cirrhosis. The cirrhotic liver is considered to be a preneoplastic organ. Therefore, chemoprevention represents a potential target in the therapeutic strategy of HCC. While pre-clinical data suggest chemopreventive effect of some drugs (28, 210), clinical studies are less convincing (211, 212). Somatostatin analogues (Octreotide and Lanreotide) are molecules that are widely used to control the symptoms in patients with endocrine and Growth Hormone producing tumours. They have also shown to have anti-proliferative activity in several tumour models (for review, see (185)). Direct anti-tumour effect is mediated through their binding with one of the 5 somatostatin receptors (sstr1-5), which are expressed with variable density among the different tumour types (180). Binding to sstr can lead to inhibition of cell cycle, inhibition of growth factors production and pro-apopotic effect (185). Somatostatin analogues also exert indirect anti-tumour activity mainly by inhibiting tumoural neoangiogenesis (213) and inhibition of growth factors release.

Studies in humans demonstrated that HCC express sstr, mainly sstr2, sstr3 and sstr5 (198-200). In rats, sstr have been shown to be expressed in activated hepatic stellate cells in CCl_4-treated animals (214), and low levels of sstr3 mRNA were found in normal liver (215). Expression of sstr is currently not known in animal models of HCC. Somatostatin analogues have shown antitumoural effects in human liver cancer cells lines *in vitro* (201, 202, 216) and in one *in vivo* animal model of HCC (217). We have previously shown that lanreotide (LAN) inhibits the formation of pre-neoplastic foci in a short-term model (218), known to induce small foci of cells that show phenotypic changes that are characteristic of neoplasia, but no signs of dysplasia. Also, these changes are completely reversible upon cessation of chemical aggression and do not lead to formal HCC (219). Now, in this study, we wanted to assess the efficacy of lanreotide in preventing *bona fide* HCC. To do so, we used the well-described model of HCC induced by diethylnitrosamine (DEN) (116). In this model, inflammation and fibrosis develop in 12 weeks and malignant tumors

within 16 weeks. As shown by others, DEN-induced tumors are genetically similar to asubclass of human HCC, with a poor prognosis (109). We tested the efficacy of LAN, whether given early, i.e. after the first injection of DEN, or when fibrosis was appearing, 11 weeks after the first DEN injection.

Materials and methods:

Animals and treatment:

Male Wistar rats (180±20 g) were purchased from Charles River Laboratories, Brussels, Belgium. They were maintained on an automatic 12 h light/dark cycle. All animals received humane care in compliance with the regulations of the Université catholique de Louvain.

All animals received, diethylnitrosamine intraperitoneally (50 mg/kg body weight dissolved in saline), once a week for 16 weeks. This regimen protocol is known to induce significant fibrosis after 12 weeks and HCC after 16 weeks. The rats were randomly assigned to one of the 3 experimental groups (n=10 per group) after the first DEN injection. A scheme of the experimental set-up is shown in Fig. 1. Lanreotide (LAN, gift from Ipsen n.v., Merelbeke, Belgium) was dissolved in saline and injected in the right thigh muscle at a dose of 3 mg/kg, every second week, according to Ipsen-Beaufour toxicology data. LAN injections started at different timepoints after the first DEN injection: either after 11 DEN injections (LAN 1116 group) or the day after the first DEN injection (LAN 0116 group). In the last group (IND), rats were administered DEN only. Food and water were accessible *ad libitum*. Animals were sacrificed after 18 weeks, allowing 2 weeks of recovery from acute necrosis after the last DEN injection. Rats were anaesthetized by ether inhalation; blood was withdrawn by aortic puncture for transaminase activity. Livers were flushed with 1 ml saline through the portal vein, to remove intra-hepatic blood, then excised and samples of anterior and posterior lobes were immersed in 4% buffered formaldehyde for histological and immunohistochemical analyses. The remaining livers were snap-frozen in liquid nitrogen and stored at – 80°C. Rats matched for age were sacrificed at the same time points and served as controls (CTL).

The count of suspicious nodules on the liver surface was performed at macroscopic examination of the liver by two independent investigators (I.B and J.A.Q) based on the following criteria: nodules with a diameter of 3 mm or more and a dysmorphic or dyschromic aspect, as previously described by many authors (70, 71, 116, 220, 221) and expressed as the number of nodules per gram of liver.

Biochemical parameters:

Serum alanine aminotransferase (ALT) were determined using automated procedures. Hepatic hydroxyproline content was determined as previously described (167).

Histology and Immunohistochemistry:

Liver sections were stained with hematoxilin-eosin according to standard procedures and analyzed by an expert liver pathologist (C. S). Liver nodules were considered as HCC at microscopy when they were composed of small hepatocytes with an increased nucleocytoplasmic ratio and a basophilic cytoplasm, gathered in thickened cell plates, with multiple mitoses present. The number of HCC per square cm of liver slide was determined independently by two investigators (I.B and C.S.) in several coded slides from anterior and posterior lobes (data are mean+SD of 2 slides per animal).

Identification of GSTp positive foci: Immunohistochemical staining was performed with an antibody raised against the placental form of rat glutathione S-transferase (GSTp; 1:100 dilution, MBL, Nagoya, Japan) revealed by En-Vision system (Dako, Denmark). Sections were counterstained with hematoxylin. For each animal, several low magnification fields (2.75 μm/pixel) that cover 1-1.5 cm^2 of the liver sections were taken at random in a representative area and digitized through a Zeiss microscope using a KS-400 system (Zeiss, Munich, Germany) coupled with a DAGE-MTI CCD72 camera (Michigan City, IN). The images were segmented by the operator in order to define GSTp-labelled foci. The ratio of stained area to total area was measured and expressed as area percentage of liver.

Fibrosis assessment: liver sections were stained with Sirius Red to visualise collagen deposition. Slides were digitized through a JVC KY-F58 colour digital camera (Japon Victor, Brussels, Belgium) and the image analyser (KS-400 system; Zeiss Vision, Munich, Germany) measured the surface ratio between the Sirius Red positive area and the total liver area.

Determination of Proliferative Index: Liver sections (5 μm thick) were stained using an anti-Ki67 antibody (1:50 dilution DAKO, Denmark) and counterstained with haematoxylin. Using a 10x magnification field, at least 2500 hepatocytes per liver were assessed for Ki67 labelling. Proliferative index was expressed as positive cells scored per 100 hepatocytes.

Evaluation of angiogenesis: Liver sections (5 μm thick) were stained using an anti-CD31 antibody (1:300 dilution, Santa-Cruz Biotechnology, Santa Cruz, CA, USA). Qualitative evaluation of the distribution of CD31 positive cells was assessed by an expert pathologist (C.S.).

Caspase 3 activity assay

Caspase 3 activity was assessed in liver homogenates using the fluorogenic substrate Ac-DEVD-AFC (Alexis Biochemicals, Lausen, Switzerland), which is cleaved enzymatically by caspase 3, releasing free AFC that produces a blue-green fluorescence.

RNA Isolation, Reverse Transcription and Real-Time Polymerase Chain Reaction:

Total RNA was prepared from the frozen liver tissue using TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA). Reverse transcription and quantitative polymerase chain reaction analysis was carried out as previously described by our group, and data normalized to the expression of RPL19 gene (222). The following primers were used: p53: sense CCATGAGCGTTGCTCTGATG, antisense TTATCCGGGTGGAAGGAAATC; p21 ^{waf1}: sense CCTGTTCCACACAGGAGCAA, antisense GATTGCGATGCGCTCATG; sstr2: sense: CTCCAACCAGACAGAGCCATACTA, antisense CGCACACCACGAAGTAGATGA; sstr3: sense: TGAATGGGAGGCTCAGTCAGA, antisense TTGGCAGTCCCTGTGCAA; sstr5: sense:

ACGTAGAGGATACGGTATGGAGGAT, antisense AGGCCGCCCACTCTTGTC; VEGFR1; sense: CGTACCCGCAACGGAGAA, antisense GCGTCCTCGGCAGTTACATC; RPL19: sense AAGCGGATTCTCATGGAACA, antisense TGGTCAGCCAGGAGCTTCTT. Normal liver was used as control (CTL), with its value set at one, except for sstr and VEGFR1, where positive controls were used (rat pituitary and peritoneum respectively), and their values set at one.

Statistical analysis:

All results are expressed as mean±SD. Data were compared two by two using the non-parametric Mann-Whitney U test, as all groups were considered with non Gaussian distribution and a number smaller than 30. A p value less than 0.05 was being considered as significant.

Results

There was no mortality during the experimental period. Animal weight was comparable in all groups, although a non-significant trend in decreased weight was observed in the LAN 0116 group (Table 1). Liver weight in LAN-treated animals was significantly less compared to IND but comparable to CTL. Liver-to-animal weight ratios were significantly increased in the IND group compared to CTL and animals treated with LAN either from week 0 or from week 11.

Somatostatin receptors are expressed in normal and cirrhotic liver:

Expression of sstr subtypes 2, 3 and 5 mRNA was assessed by RT-qPCR in normal liver and the livers from IND and LAN-treated animals. Results are shown in Table 2. Compared to rat pituitary, expression of all sstr 2, 3 and 5 was very low in normal and diseased liver. Expression was the most important for sstr3, followed by sstr5, than sstr2, either in normal liver, in line with previous results (215), but also in cirrhotic and tumoral tissue. Compared to normal liver, sstr3 expression was significantly increased in IND and in LAN-treated animals. No statistical difference was observed between IND and LAN concerning sstr3, while sstr2 and sstr5 were more expressed

in LAN 1116 compared to IND. Unfortunately, due to unfit antibodies, confirmation and localisation of sstr proteins in the liver could not be performed by immunohistochemistry.

Lanreotide prevents DEN induced dysplastic nodules and HCC.

As illustrated in Figs 2a, DEN induced a macronodular aspect and several large tumors (Fig. 2a: A, D and E); LAN, either started early or after 11 weeks of DEN, dramatically changed the macroscopic aspect of livers (Fig. 2a: B, C), with a smooth surface when started early, and a subtle granular aspect when given at week 11. On histology (Fig 2b), DEN completely modifies the liver architecture. A lot of nodules of varying sizes are present, delineated by fibrous septae. Numerous nodules are composed of large hepatocytes, with an abundant clear or, more frequently, eosinophilic cytoplasm and atypical, bizarre nuclei (Fig.3A). These nodules represent the classical foci of altered hepatocytes (FAH) described in the literature. These FAH were also observed in the LAN-treated livers. Less frequently, smaller nodules were found, with more regular, less modified hepatocytes. Finally, HCC were found, with characteristics depicted in the Material and Methods section, of small (Fig.3b) or medium size (Fig.3c), but undoubtfully malignant cellular phenotype (Fig. 3d).

The density of macroscopic dyschromic nodules present on the liver surface, according to the established criteria (116), was significantly lower in LAN-treated livers than in IND (Fig.4a; $p<0.01$). When analysed for pathologic criteria of malignancy, not all dyschromic nodules proved to be HCC, unlike what was observed by Schiffer et al. (116). Therefore, we performed a thorough analysis of several regions of both anterior and posterior lobes of the liver, and quantified criteria-established HCC per square cm of liver surface (Fig.4b). In the IND group, we found 2.6±0.6 HCC vs 1.4±0.4 (LAN 1116, $p<0.01$) and 0.6±0.7 (LAN0116, $P<0.01$). Thus LAN, whether given at the beginning of the carcinogenic process or later on, when fibrosis is present, reduces the incidence of HCC in this model.

GSTp is a marker of cell early phenotypic transformation, widely used in rat models of carcinogenesis (119) and whose expression is co-regulated by genes associated with cellular proliferation and transformation (125). Parallel to macroscopic tumor assessment, GSTp expression was very high in the IND group, with up to 78±7% of the liver surface being positive (Fig 5). In LAN-treated animals this expression was lowered by a factor 2, with 40±20% positive cells in the LAN 0116 group ($p<0.001$), and to 35±20% of positive staining in the LAN 1116 ($p<0.001$). In our model, GSTp staining was positive in FAH (Fig.3A) and large HCC (Fig.2a: E) but negative for small and medium-sized HCC (Fig 3B, C), so we considered it as an indirect marker of cell malignancy.

Lanreotide decreases ALT levels and fibrosis induced by DEN:

DEN induced increased ALT serum levels compared to CTL ($p<0.05$) and LAN-treated animals ($p<0.01$) (Table 1). As evaluated by morphometric analysis of Sirius Red stained slides, there was significantly less fibrosis in LAN-treated livers than in IND livers. This was confirmed by decreased hydroxyproline content in LAN treated livers ($p<0.001$) (Fig. 6b).

Thus, in accordance with previous reports (116), DEN induces cirrhosis and HCC. Lanreotide significantly decreased fibrogenesis induced by DEN, as well as HCC, even when LAN was given late in the carcinogenic process.

Lanreotide inhibits hepatocyte proliferation and interacts with the p53 pathway.

As shown in Fig. 7, the index of proliferation (ratio of positive Ki67 cells/total cell count), was significantly increased in the livers of the IND group, with a value of 21.7+/-3% of KI67 positive hepatocytes, compared to normal liver (1±0.5%; $p<0.05$), in line with our previous results (218). LAN treatment significantly decreased the level of proliferation, to a Ki67 index of 8.7+/-2% in the LAN 0116 group ($p<0.01$) and to 10.6+/-3%, in the LAN 1116 group ($p<0.01$). mRNA levels of p53 and of its downstream effector $p21^{waf1}$ significantly increased by a factor 6 in the IND group

compared to normal liver ($p<0.05$). In LAN-treated animals, liver p53 mRNA was down-regulated, but $p21^{waf1}$ mRNA remained as elevated as in IND; mRNA of p27 were not significantly different compared to normal liver, in all experimental groups (data not shown).

Lanreotide, when given early, induces significant apoptosis in cirrhotic liver.

We performed a caspase-3 assay to assess apoptosis, through the activity of this important effector caspase. DEN (IND group) did not induce a significant caspase 3 activity compared to normal liver (Fig. 9). By contrast, LAN treatment, when given early, was associated with a significant 3 times increase in caspase-3 activity ($p<0.01$). This was not observed when LAN is given at a later timepoint. So we suggest that apoptotic features are induced only when LAN is given very early in the carcinogenic process.

Inhibition of angiogenesis is observed when lanreotide is given at the beginning of the carcinogenic process, through inhibition of the VEGF receptor subtype 1, Flt-1.

To analyse angiogenic features, we targeted the cell surface CD31 antigen, also called PECAM-1, for Platelet/endothelial cell adhesion molecule, using immunohistochemistry. In normal liver, various cell types express CD31, with homogenous distribution through the liver. We observed a strong staining in the IND group, with linear distribution along the cell plates, suggesting neo-vascularisation. In the LAN 1116 group, these patterns suggestive of neo-vessels were less present, and disappeared in the LAN 0116 group. Because Flt-1 was shown to be the main VEGF receptor subtype overexpressed in a rat model of cirrhosis using DEN (114), we quantified its mRNA expression in the different experimental groups, using rat peritoneum that was wounded with LipoPolySaccharide as positive control. Results, shown in Fig 10, indicate that DEN induced a doubling of Flt-1 expression compared to normal liver. More interestingly, LAN, when given early, completely inhibited Flt-1 expression while administration of LAN after 11 weeks of DEN decreased Flt-1 expression induced by DEN to those of controls.

Discussion:

Chemoprevention of HCC remains an important challenge in the therapeutic strategy of this deadly disease. The rationale supporting the proposal of chemoprevention in this setting is that HCC develops on a diseased liver, i.e. cirrhosis, which is diagnosed well before HCC. Attempts to prevent HCC when cirrhosis is installed have however not been very convincing up to now. We previously showed in animal model, using DEN and acetylaminofluorene in sequential fashion, that LAN could slow down the onset and progression of very early foci of altered hepatocytes, showing phenotypes of transformed preneoplastic cells (218). However, in this previous model the chemicals used did not induce neither dysplasia nor neoplasia.

In the present work, we evaluated whether LAN could prevent HCC. For this purpose, we used a carcinogenic model, elaborated by Schiffer et al., that uses DEN, a mutagen that alters DNA by giving rise to alkylating nucleophilic sites, and induces HCC in 100% of male Wistar rats (116). We administered LAN at two different times to test the following hypotheses: Could LAN inhibit the occurrence of HCC when given before liver aggression (LAN 0116 group), or when established inflammation and fibrosis are already present (LAN 1116 group)?

Indeed we could show, that whether given very early, i.e. 24 hours after the first DEN injection, or later on, after 11 weeks of DEN administration, LAN could inhibit tumor development. Quantitative tumor analysis was addressed by three parameters: firstly, the nodule-to-liver weight ratio. However, unlike others, we found that not all nodules where HCC, but a significant ratio was composed of dysplastic nodules. So we decided to evaluate the incidence of HCC in liver sections, defined by precise, well-established histological criteria. This analysis did show similar findings, i.e. a drop in the number of tumors in the LAN-treated groups, by at least 50%. Third, we assessed the GSTp expression level, that also decreased by a factor 2 in treated groups compared to IND, suggesting that here, as in many papers (reviewed in (119)) GSTp is a good marker of preneoplasia and neoplasia. Finally, a more indirect parameter is the liver-to-body weight ratio that is often

elevated in case of HCC presence (223), that showed here to be significantly higher in the IND group compared to CTL, and very close to CTL values in the treated groups.

We could show that sstr were present in all 3 experimental groups, although in small amounts compared to our positive control, rat pituitary. A decrease of sstr3, which was the subtype that seems to be most present, was observed in the LAN-treated groups, although not significantly. This observation is in line with the known down-regulation of sstr expression observed when patients are exposed to chronic exposure to Somatostatin analogues.

In agreement with what we showed in a previous work (218) and *in vitro* data (202), we demonstrated that the tumor growth inhibitory effect of LAN was due to inhibition of proliferation of hepatocytes. p53 is a well known tumor suppressor gene activated by DNA damaging (224). The production of p53 protein leads to the activation of DNA repair or, when impossible, to apoptosis (225). p53 mRNA was very much induced in the IND group, which is contradictory to some data from the literature that show a decreased p53 expression in foci of altered hepatocytes compared to surrounding liver (226). It must be stressed that the model used by the authors was comparable to the short-term model we used in previous experiments (218), but definitely different to the one used in the present experiments. Here, it is possible that overexpression of p53 and its effector p21 were related to the cellular stress induced by DEN, reflected by the inflammatory/fibrotic environment, the presence of cell necrosis and proliferative hepatocytes. Indeed, it is known that p53 is overexpressed when cirrhosis and HCC coexist in patients (227) but also during normal rat liver regeneration, as shown by our group (228). As we found a decreased p53 expression in LAN-treated groups, it is tempting to say that this was due to the decreased inflammation and fibrosis in those groups, leading to less reactive p53 activation, but also that a putative p53-driven proliferative stimulus found in DEN-induced livers, is inhibited by LAN. mRNA of p27 was not significantly different compared to normal liver, in all experimental groups (data not shown), in line with the results we showed in the short-term carcinogenic model (218) and *in vitro* data (148).

Somatostatin and its analogue octreotide have demonstrated anti-fibrotic properties in

different models of experimental fibrosis in rats (229-232) Here, we demonstrated for the first time that LAN significantly decreases fibrosis in an animal model. This was accompanied by a decrease in serum ALT level, suggesting less hepatocyte necrosis. More interestingly, this effect on fibrosis and necrosis was observed also in the LAN1116 group, suggesting that even when given very late in the process, LAN could reverse the situation. It is known that rat hepatic stellate cells (HSC) express sstr (214). So one possible explanation could be that LAN directly inhibits the collagen production by HSC, in line with previous work (233). Another alternative explanation could be that LAN interfered with the metabolism of DEN and may have reduced its toxicity. However we believe this is unlikely because the antifibrotic effect was also observed in rats injected with DEN for 11 weeks before receiving the first LAN injection. Furthermore, DEN mutagenic effect is driven through its active metabolites, which formation is mainly dependent upon CYP450 2E1 or CYP2A6 activity (234, 235). To our knowledge, LAN does not interfere with CYP2E1 or CYP2A6 (236).

Octreotide is known to have anti-angiogenic properties (196). Here we could see a capillarization of liver parenchyma in livers induced by DEN compared to normal liver. A normalization of CD31 staining was observed in LAN-treated animals (data not shown). Flt-1, the main VEGF receptor that is known to be overexpressed in cirrhosis in humans (237) and rats (114), was dramatically decreased by LAN when given early, compared to IND and normal liver. This decrease was not observed in the LAN 1116 group, suggesting that anti-angiogenic effect of LAN can be obtained after a long exposure only.

Apoptosis is another important key player in cancer control. In tumoral cell lines, somatostatin analogues have shown to induce apoptosis upon binding to sstr2 and sstr3 (192, 238). Here we show that LAN induces significant apoptosis only when given early. So the anti-apoptotic effect of LAN probably accounts only marginally for the anti-tumoral effect observed, probably partly because LAN has less binding affinity for sstr3 compared to sstr2 (239), which was much less expressed here.

In conclusion, we showed that LAN decreases significantly the number of HCC in an animal sequential model, inducing cirrhosis followed by cancer. This effect is maintained even when the somatostatin analogue is given at later time-point in the carcinogenic-fibrogenic process. Our results strongly suggest that inhibition of cell proliferation and slowing down of fibrogenesis, are the main effects for the anticancer properties of LAN. Antiangiogenesis and stimulation of apoptotis contributes probably less to this effect since it was observed only when LAN was given at the very beginning of the carcinogenic process. These results warrant further investigations in more simple situations, such as cell culture, to analyse causative factors. In particular, the anti-fibrotic mechanisms could be dissected by looking *in vitro* to the effect of LAN on HSC.

Aknowledgements:

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Conflict of interest statement:

None declared

Table 1

	CTL	IND	LAN 0116	LAN 1116
Animal body weight (gr)	462±19.3	445±61	405±66	438±48
Liver weight (gr)	14.17±0.2	19.97±4.53	11.6±2***	14.07±1.39**
Liver to rat weight ratio (%)	3.07±0.09	4.24±0.74$	2.9±0.49***	3.25±0.45**
ALT (U/L)	76.7±3.1	257±50 $	83.6±16.3**	114.4±28**

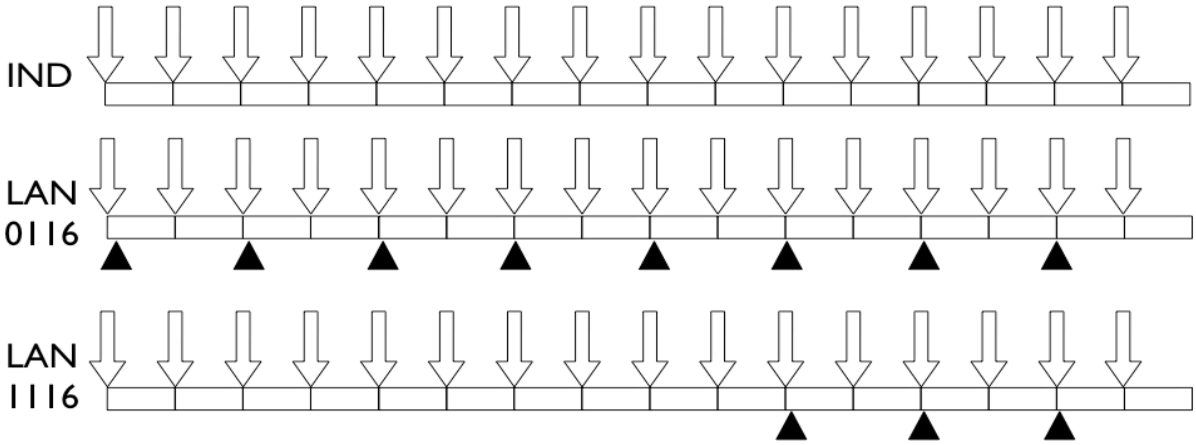
Animal body weight (in grams), liver weight (in grams), ratio of liver-to-animal weight (in %) and Alanine Transferase levels (ALT, units/liter) in normal rats (CTL), in rats treated with lanreotide for 6 weeks (LAN 1116) or 16 weeks (LAN 0116) and in rats receiving DEN only (IND). Data are mean±SD. ($): $p<0.05$ for IND vs CTL; (*): (**): $p<0.01$ for IND vs LAN; (***): $p<0.001$ for IND vs LAN

Table 2

	NL liver	IND	LAN 0116	LAN 1116
sstr2	ND	0.00029±0.00005	0.0012 ±0.0004**	0.025 ±0.026**
sstr3	0.014 ± 0.009	0.92 ±0.87 $	0.16 ± 0.11 $$	0.25 ± 0.12$$
sstr5	0.006±0.004	0.006±0.012	0.0036±0.001	0.14±0.13**

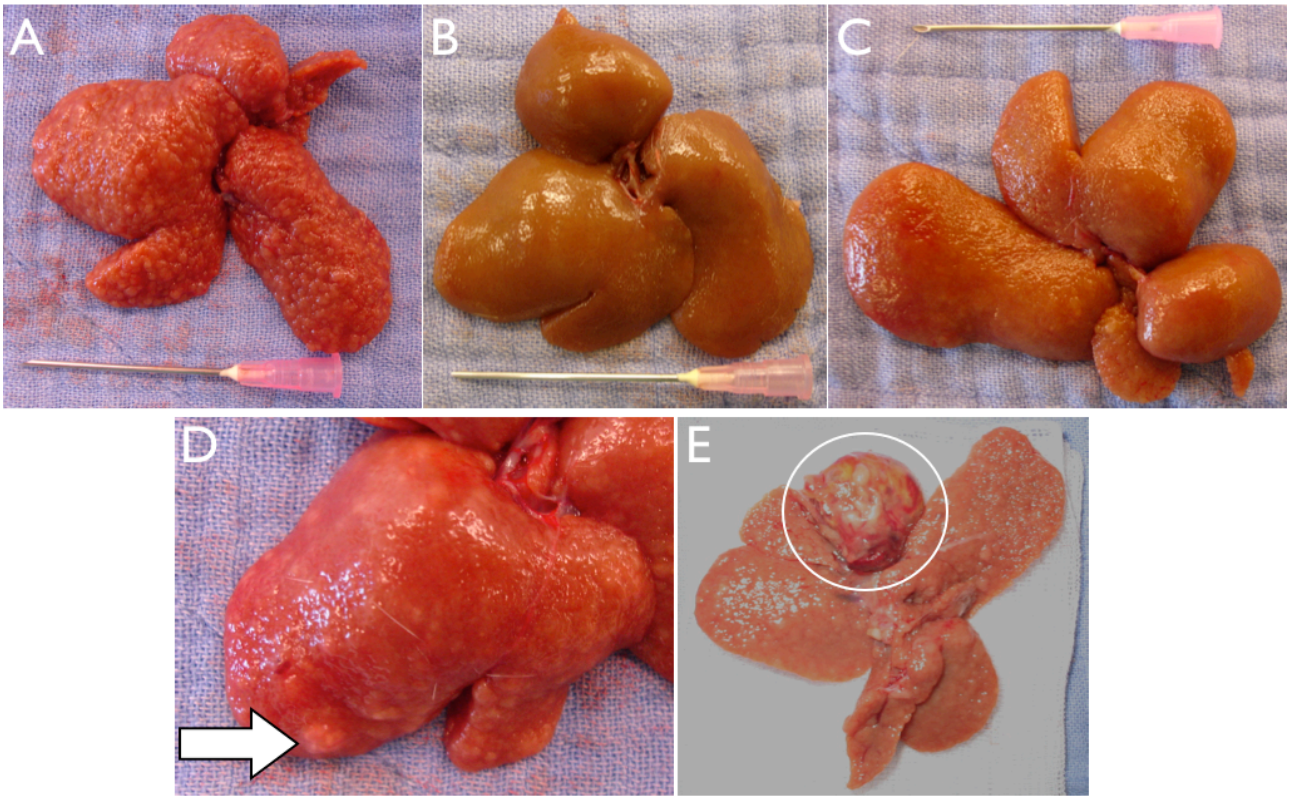
Expression of somatostatin receptor subtype (sstr) 2, 3 and 5 mRNA, by RT-qPCR, in %. Values are shown for normal liver, in rats injected with DEN only (IND), DEN+LAN from week one to 16 (LAN 0116), DEN+LAN from week 11 to 16 (LAN 1116). For all experiments, rat pituitary was taken as positive control, with its value set at 100%. ND means Not Detectable. ** means $p<0.01$ for LAN vs IND; $ means $p<0.05$ for IND vs NL liver; $$ means $p<0.01$ for LAN vs NL liver.

Figure 1



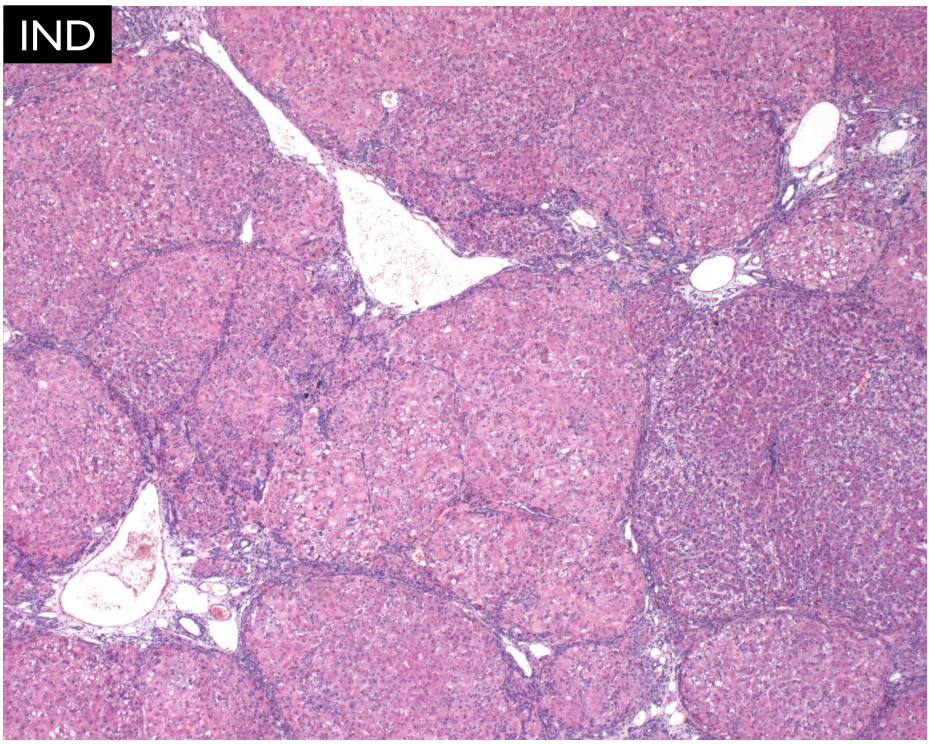
Scheme of the experimental model. All animals received the same carcinogenic drug, i.e. DEN (white arrows), given intraperitoneally at a dose of 50 mg/kg body weight dissolved in saline. This dose was given every 7 days for 16 weeks. The rats (n=30) were randomly assigned to one of the 3 experimental groups after the first DEN injection. Lanreotide (black arrowheads) was dissolved in saline, injected in the right thigh muscle at a 3 mg/kg concentration every 14 days, starting at different times after the first DEN injection: either after 11 DEN injections (LAN 1116 group, n=10) or directly after the first DEN injection (LAN 0116 group, n=10). In the last group (IND, n=10), rats were administered DEN only. A group of 3 rats received no treatment and served as control (CTL), not showed.

Figure 2a

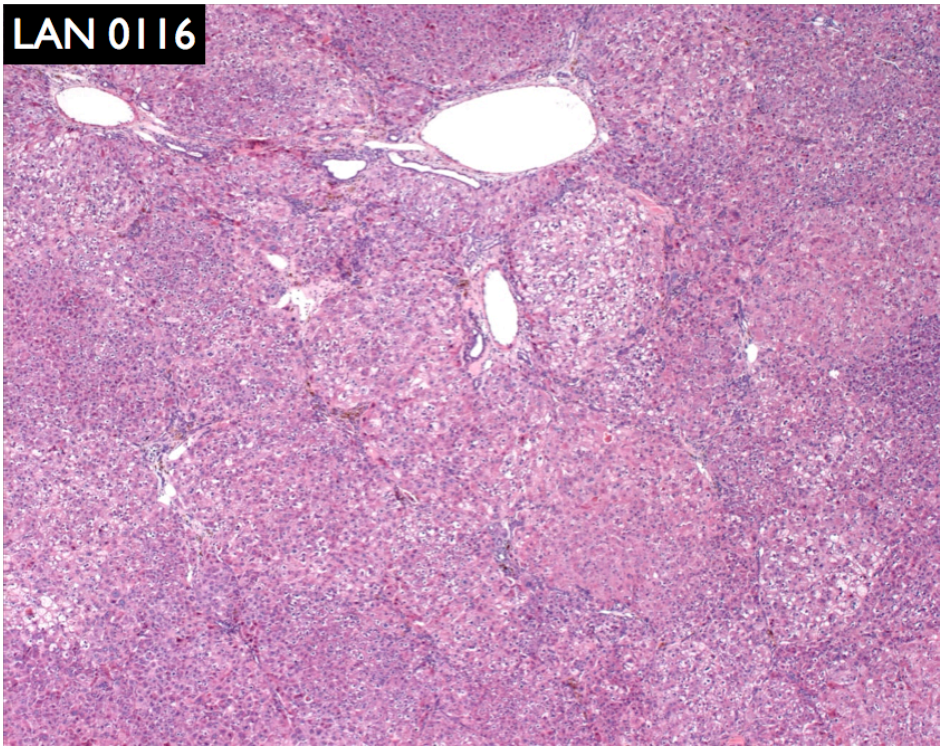


A. Macroscopic appearance of the liver in the animal injected with DEN for 16 weeks.
B. Macroscopic appearance of livers in the group treated with LAN from week one to 16.
C. Macroscopic appearance of livers in the group treated with LAN from week 11 to 16. Note the finely granular aspect of the liver surface, compared to the smooth surface of the liver in the LAN 0116 group.
D. Macroscopic appearance of a suspicious nodule, being > 3mm, white and protrubing on the liver surface.
E. Macroscopic appearance of a tumor, proven HCC on histology.

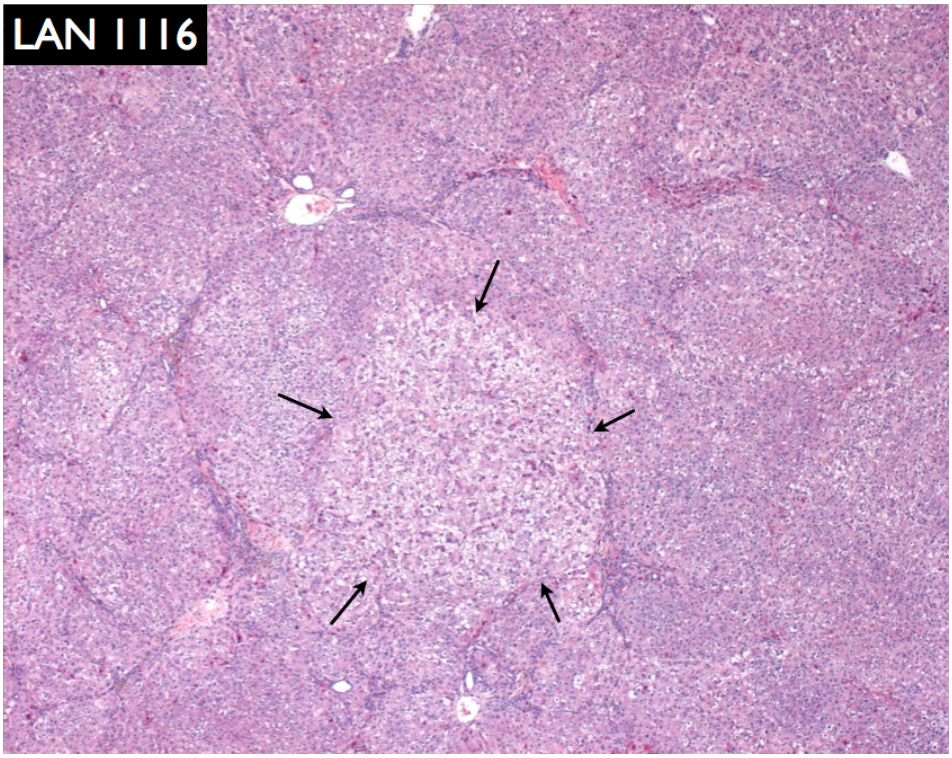
Figure 2b



Histological appearance of the liver in the DEN-injected animals. H&E staining, magnification 25x

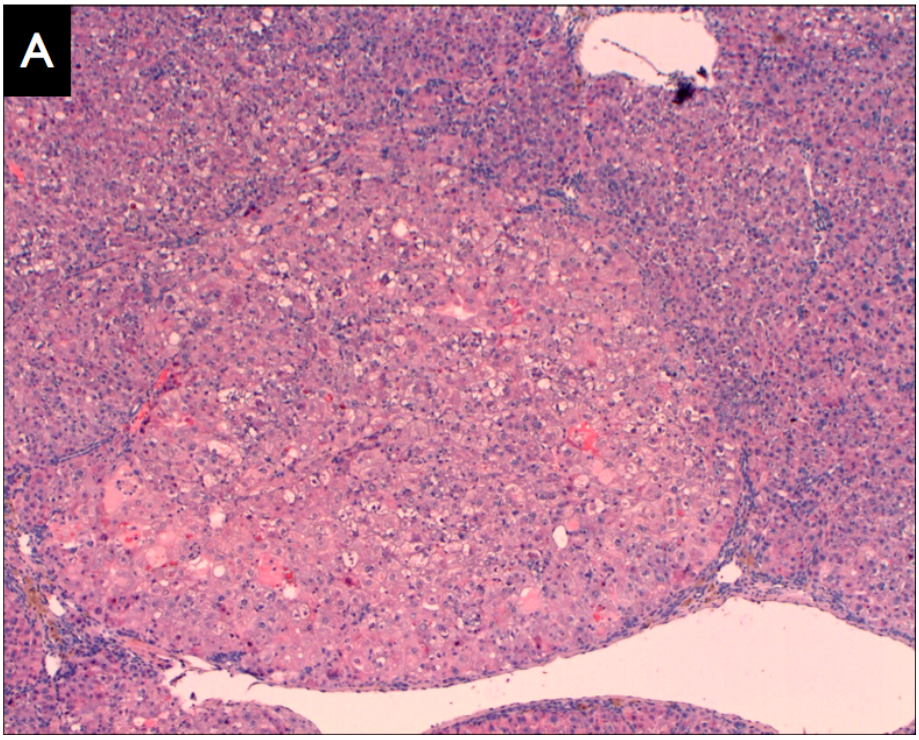


Histological appearance of the liver in the animals injected with DEN for 16 weeks and treated with LAN from week 1 to 16. Note that the nodular aspect is not present. H&E staining, magnification 25x

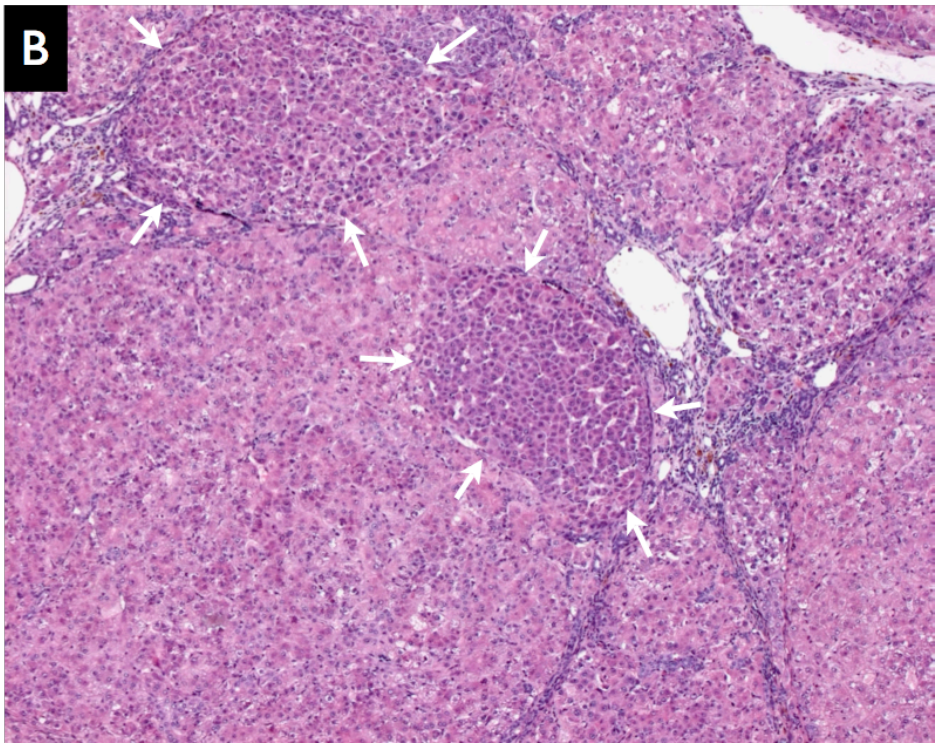


Histological appearance of cirrhotic liver in the animals injected with DEN for 16 weeks and treated with LAN from week 11 to 16. Note the discrete nodular aspect, with a focus of altered hepatocytes (FAH). H&E staining, magnification 25x

Figure 3

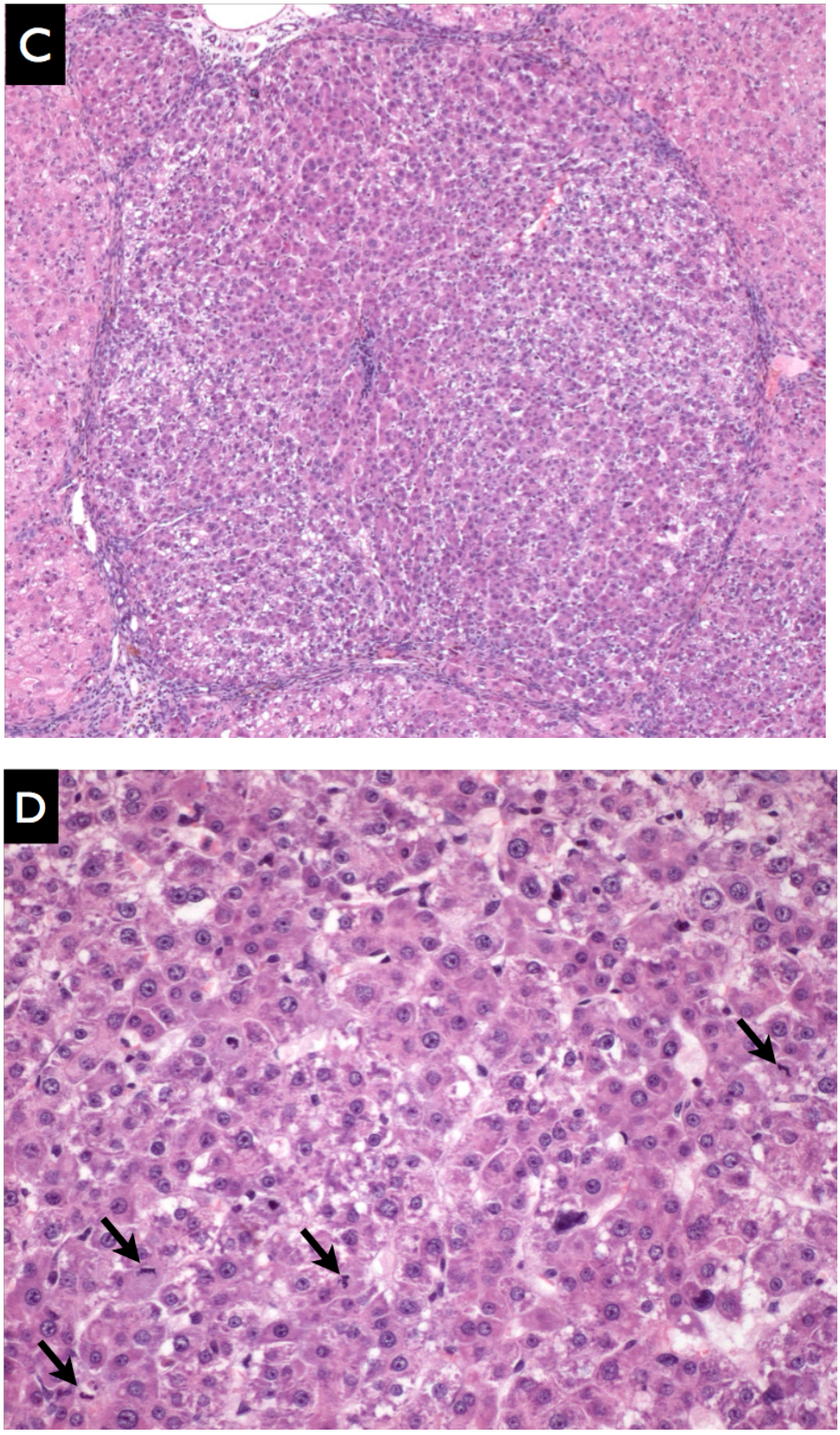


A: Histologic appearance of a focus of altered hepatocytes (FAH). Note the clear delineation, the pale or eosinophilic cytoplasm, the big nucleocytoplasmic ratio and some apoptotic bodies. H&E staining, magnification 50X



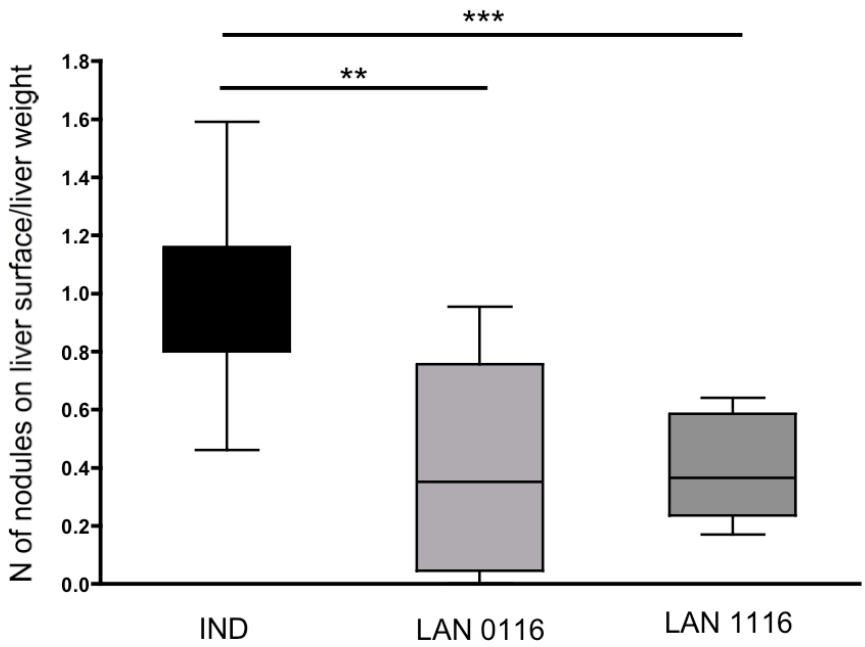
B: Highlighted by arrows, two small HCC, characterized by small hepatocytes with an increased nucleocytoplasmic ratio and a basophilic cytoplasm, gathered in thickened cell plates. H&E staining, magnification 50X

Figure 3



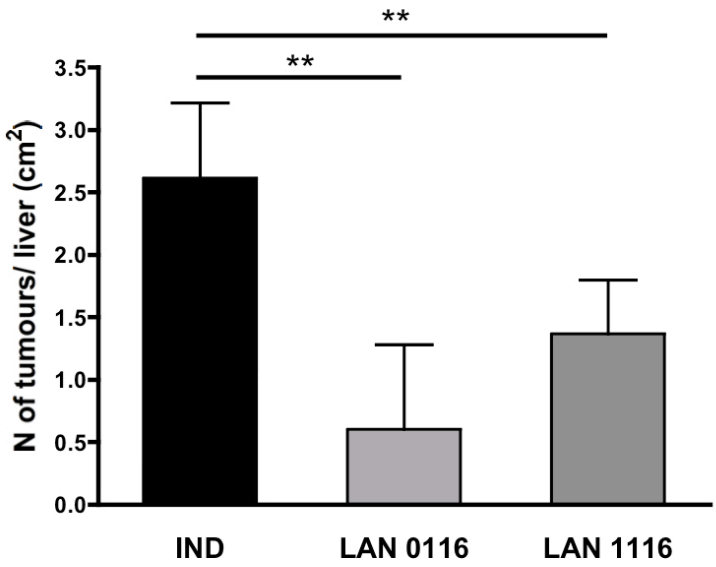
C and D: medium size HCC, with the same characteristics. In D, please note the mitoses (arrows). H&E staining, magnification 50x (C) and 100X (D)

Figure 4



(**): p<0.01; (***): p<0.001

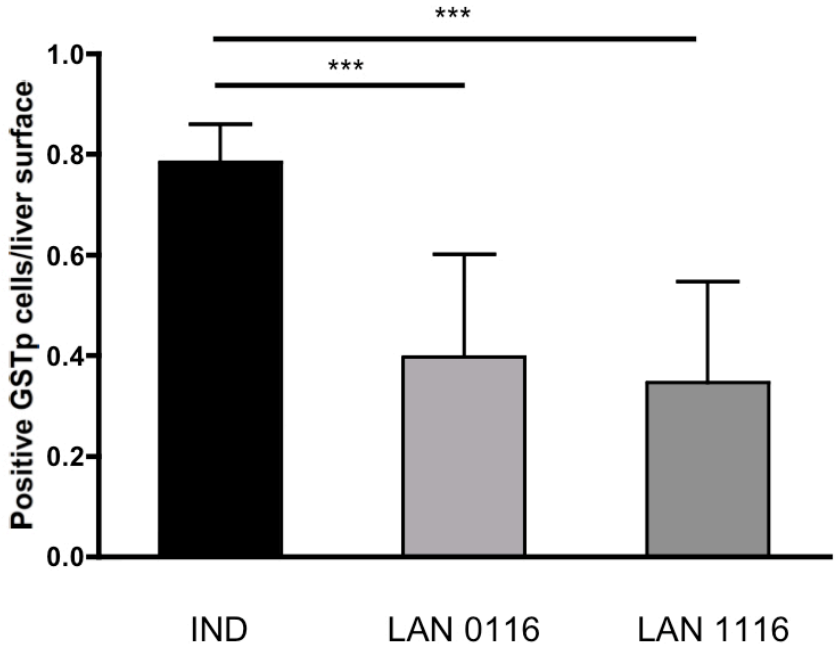
Figure 4a: Histogram depicting the mean+SD ratio of the number of sub-capsular suspicious nodules per liver weight in lanreotide-treated groups, either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116) and in the induced group (IND). (**): p<0.01 for IND vs LAN 0116; (***): p<0.01 for IND vs LAN 1116.



(**): p<0.01

Figure 4b: Histogram depicting the mean+SD number of hepatocellular carcinoma per square cm of liver slide, evaluated independently by to investigators in anterior and posterior slides, in lanreotide-treated groups, either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116) and in the induced group (IND). (**): p<0.01 for IND vs LAN 0116 and LAN 1116.

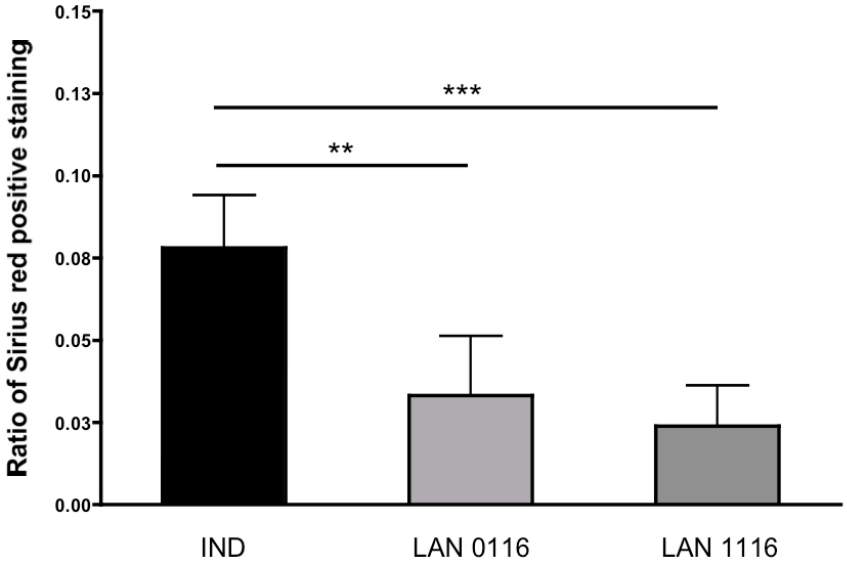
Figure 5:



(***): p<0.001

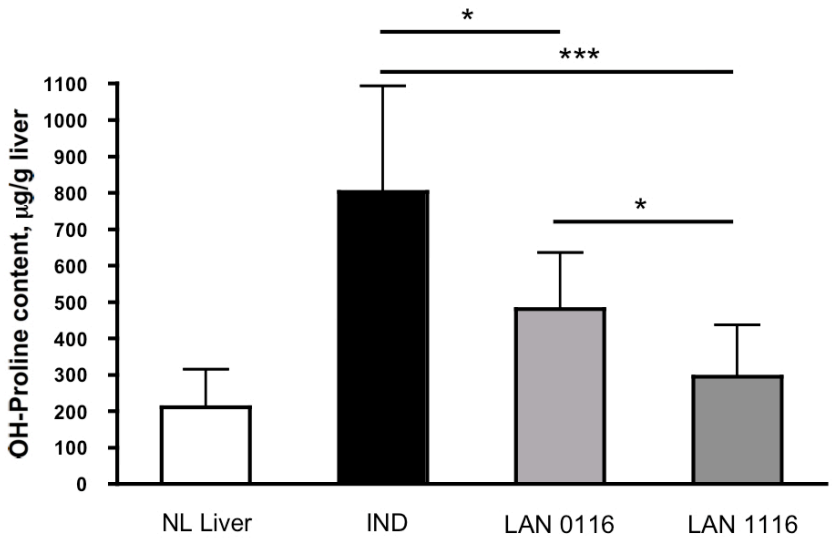
Histogram representing GSTp expression, determined by immunohistochemistry, as % of the liver slide surface in LAN-treated groups, either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116) and in the induced group (IND). (***): p<0.001 for IND vs LAN 0116 and LAN 1116.

Figure 6:



(**): p<0.01; (***): p<0.001

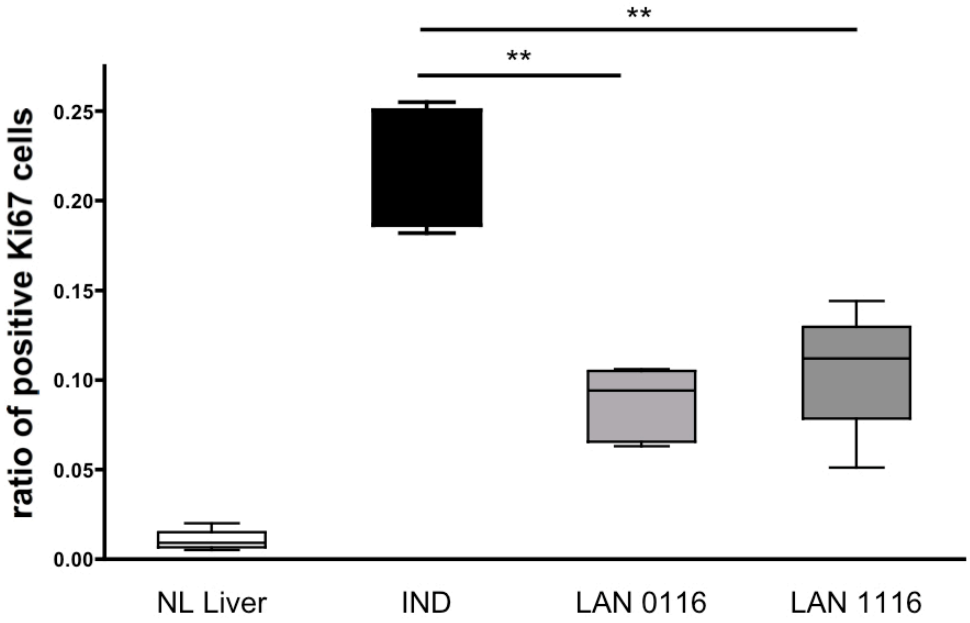
Figure 6a: Graph representing Sirius red expression, determined by immunohistochemistry and quantified by morphometry, as % of the liver slide surface, in LAN-treated groups either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116), and in the induced group (IND). (**): p<0.01 for IND vs LAN 0116; (***): p<0.001 for IND vs LAN 1116



(***): p<0.001 (**): p<0.01; (*): p<0.05

Figure 6b: Hydroxy-proline content, expressed as µg/g liver weight in normal liver (CTL), taken as reference, in the LAN-treated groups either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116), and in the induced group (IND). Data are mean+SD. (*): p<0.05 for IND vs LAN 0116 and for LAN 0116 vs LAN 1116; (***): p<0.001 for IND vs LAN 1116

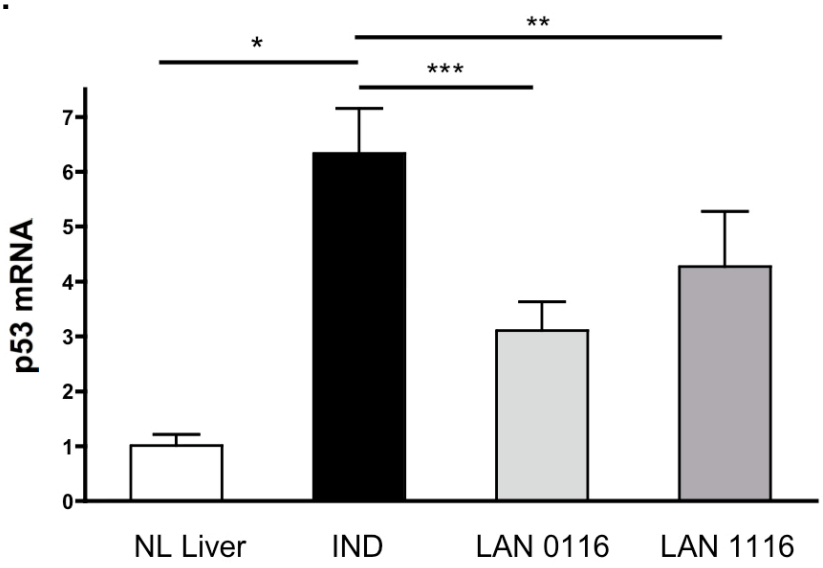
Figure 7:



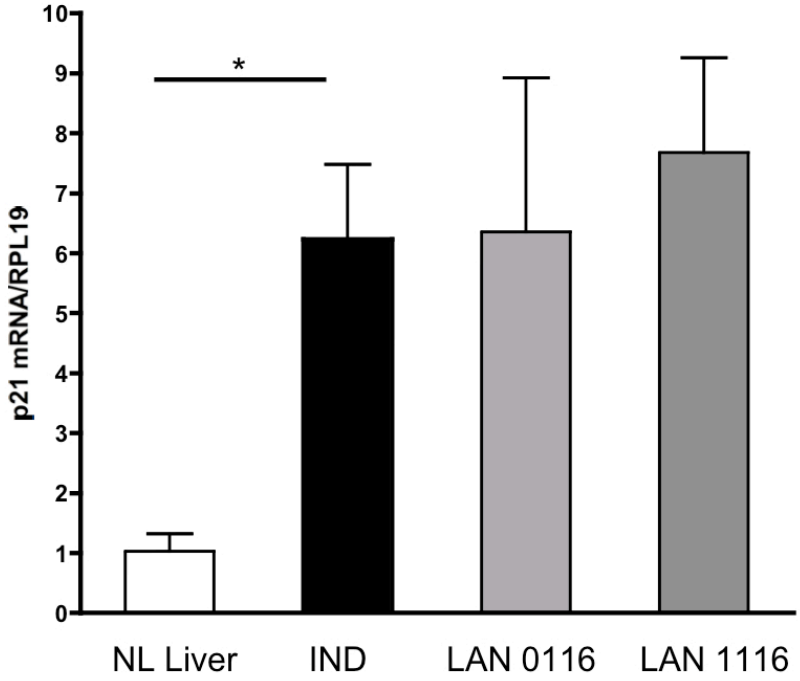
(**): p<0.01

Percentage of hepatocyte nuclei labeled with the marker of proliferation Ki67, in LAN-treated groups either for 6 weeks (LAN 1116) or for 16 weeks (LAN 0116), and in the induced group (IND). Data are mean±SD (**): p<0.01 for IND vs LAN 0116 and LAN 1116.

Figure 8:



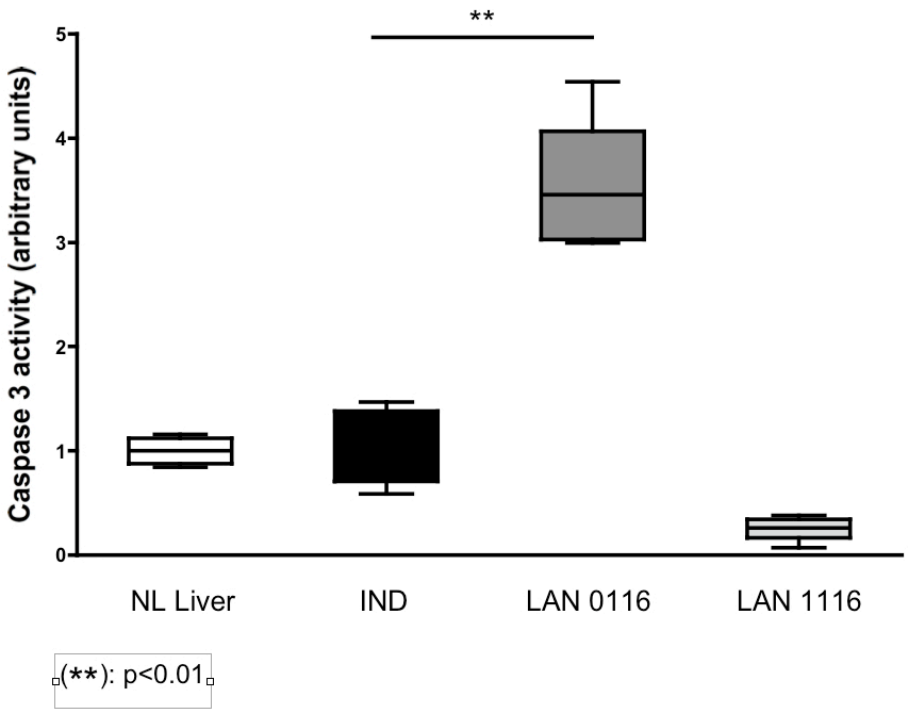
(*): p<0.05; (**): p<0.01; (***): p<0.001



(*): p<0.05

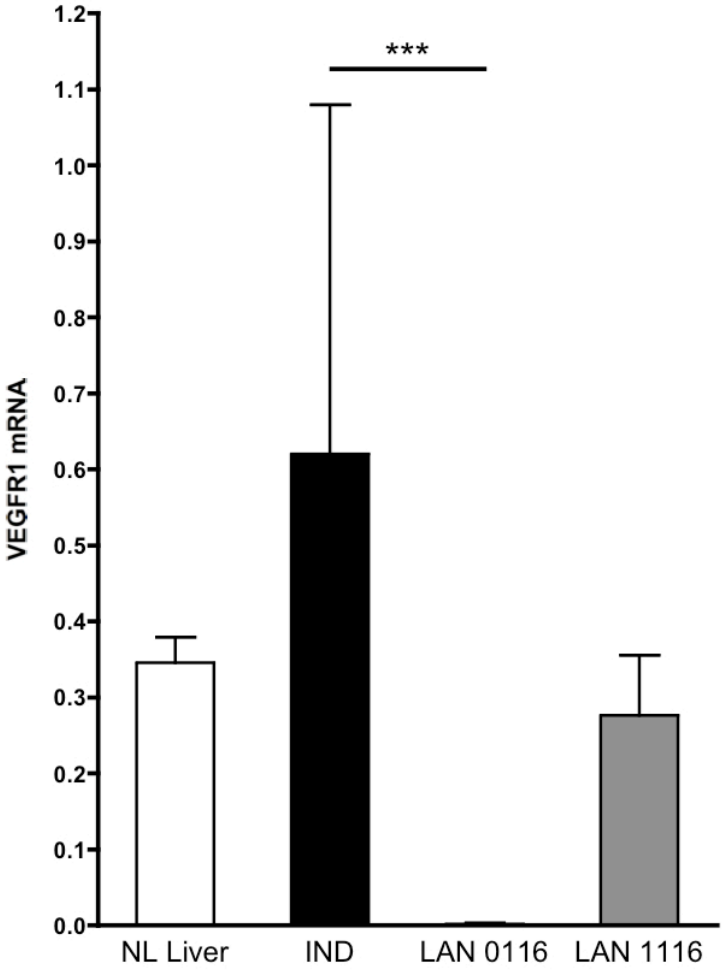
Graphs representing mRNA expression of (a) p53 and (b) $p21^{waf1}$ and in arbitrary units (i.e. values normalized to respective RPL19 values) in normal liver taken as control, with value set at one, in LAN-treated groups, either for 16 weeks (LAN 0116) or for 6 weeks (LAN 1116) and in the induced group (IND). Data are mean+SD. (*): p<0.05 for IND vs CTL (8a, 8b); (**): p<0.01 for IND vs LAN 1116; .(***): p<0.001 for IND vs LAN 0116.

Figure 9:



Expression of activated caspase 3, in arbitrary units, in normal liver taken as control (CTL), with its value set at one, in LAN-treated groups either for 16 weeks (LAN 0116) or for 6 weeks (LAN 1116), and in the induced group (IND). Data are mean±SD. (**): $p<0.01$ for IND vs LAN 0116.

Figure 10:



Graph representing mRNA expression of Flt-1, in arbitrary units (i.e. values normalized to respective RPL19 values) in rat peritoneum taken as control, with value set at one, not shown on the graph, in LAN-treated groups, either for 16 weeks (LAN 0116) or for 6 weeks (LAN 1116) and in the induced group (IND). Data are mean+SD. (***): $p<0.001$ for IND vs LAN 0116

Figure 1:

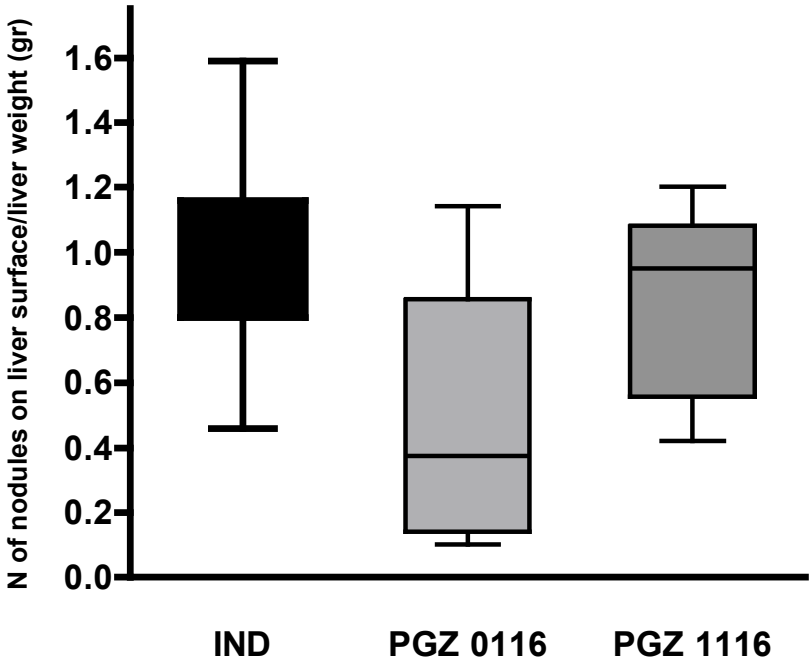
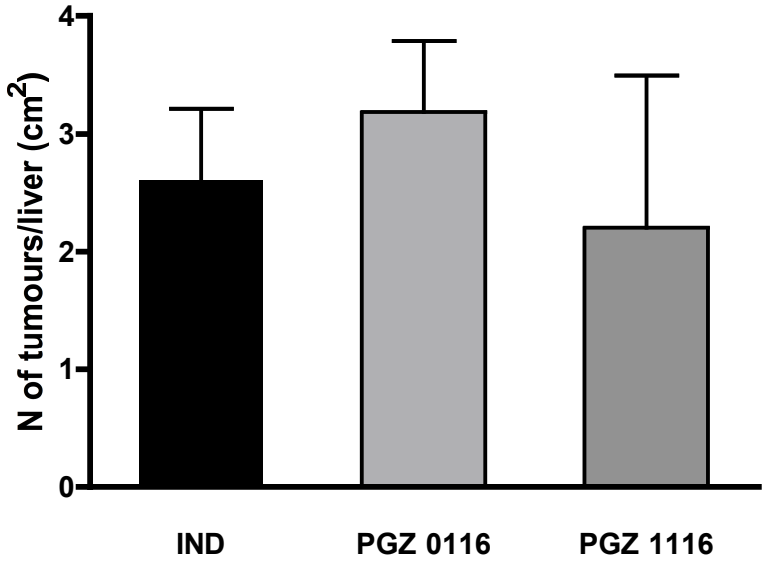


Figure 2:



VI. Pioglitazone does not prevent the appearance of hepatocellular carcinoma induced by diethylnitrosamine in the rat liver.

In this study, we wanted to evaluate the effect of PGZ in the protocol. The following results were obtained: first, only 4/10 rats survived the experiment when receiving PGZ from week 1 to 16, and 9/10 when receiving PGZ from week 11 to 16. The surviving rats in the group given PGZ from week 0 were significantly leaner compared to rats receiving DEN only: 305 vs 437 gr ($p<0.01$).

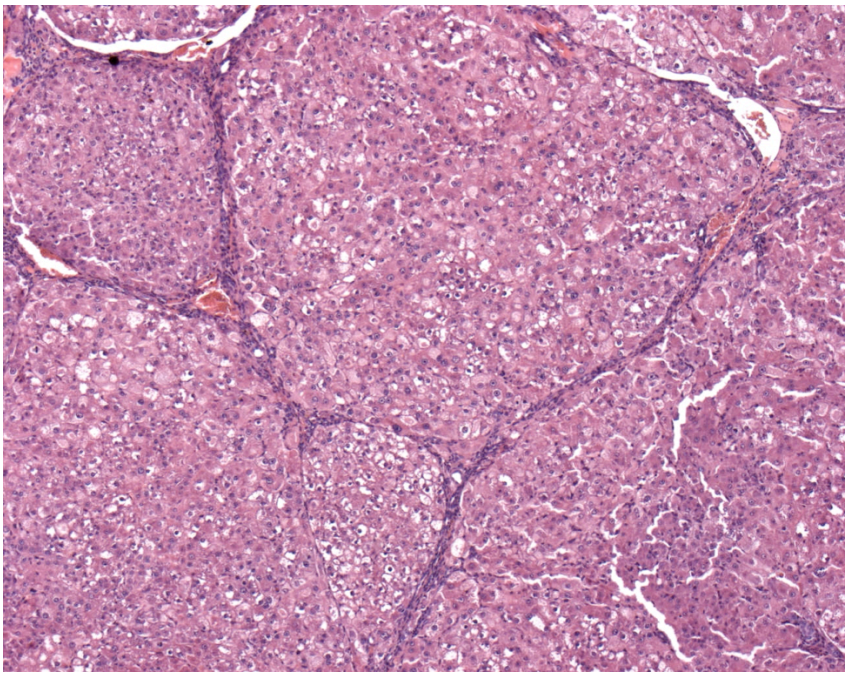
The number of dyschromic nodules found at the liver surface per liver weight (Figure 1) were not statistically different between groups, although there seemed to be a trend in less nodules for the PGZ 0116 group (PGZ from week 1 to 16), where only 4 rats survived.

The number of HCC per liver cm^2 was elevated in both the PGZ 0116 and 1116 groups, not different from the IND group (Figure 2).

PGZ did not seem to reduce fibrosis, as shown by Figures 3a (PGZ 0116) and 3b (PGZ 1116); fibrosis was thus not quantified by morphometry or hydroxyproline content.

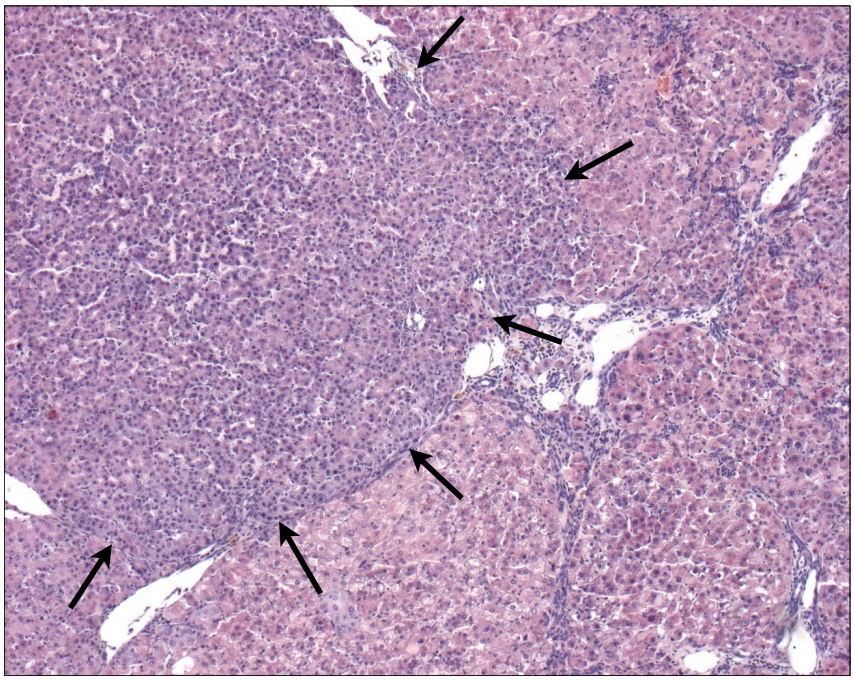
From these results we concluded that although PGZ was effective in a model inducing preneoplasia, it was not successful in preventing the appearance of HCC.

Figure 3a:



Histological appearance of the liver in the animals injected with DEN for 16 weeks and treated with PGZ from week 1 to 16. H&E staining, 50x

Figure 3b:



Histological appearance of the liver in the animals injected with DEN for 16 weeks and treated with PGZ from week 11 to 16. H&E staining, 50x. Note the presence of lesion characteristic of HCC, depicted by black arrows.

VII. Conclusions:

The rationale for chemoprevention in hepatocellular carcinoma is obvious. The disease is frequent, the population at risk can be identified and once the tumour is present, the prognosis is poor.

We chose to evaluate the efficacy of two compounds. Pioglitazone, a PPARγ agonist, used in clinical practice for type 2 diabetes, shows an excellent safety profile. It has been shown to reduce liver fibrosis in rat models (163) and also in humans with NASH (168). It also induces cellular differentiation, an important property in chemoprevention. Lanreotide, a synthetic analogue of somatostatin, has also shown a good toxicity profile in the clinical setting. SMS analogues have shown anti-neoplastic effects in many cancer types (185) and anti-fibrotic effects in rats (231).

The animal models used in the present work address two different questions:

- First, to test their effects in primary prevention, can PGZ and LAN inhibit very early changes in liver carcinogenesis? To answer, we induced foci of altered hepatocytes (FAH) in rats in a sequential way, using DEN and AAF as initiator and promoter of preneoplastic hepatocytes.
- Second, in the setting if significant fibrosis, in secondary prevention, can the drugs prevent the appearance of HCC? For this, we induced fibrosis and malignant tumours in rats by using DEN.

We demonstrated that pioglitazone is decreasing FAH in the model of early carcinogenesis, mainly by inhibiting cell proliferation, through interference with cyclins and cyclin inhibitors. We thus confirmed *in vivo* the results observed by authors using pioglitazone in *in vitro* models (148, 153). However, pioglitazone was unsuccessful in preventing cirrhosis and the onset of and dysplastic nodules, as observed macroscopically

in the second model used; therefore we chose to explore no further the effect of pioglitazone in HCC.

Lanreotide was able to decrease FAH effect in the early carcinogenesis model, by acting both on proliferation and apoptosis of the altered hepatocytes. Furthermore, it also had effect in a model of more advanced carcinogenesis, with established cirrhosis and HCC. In this context, lanreotide decreased significantly the number of HCC and, interestingly, also the fibrosis induced by DEN given weekly for 16 weeks in male rats.

Liver carcinogenesis is a highly complex process, involving many different pathways and, up to now, existing animal models, including the one we used, have not approached this complexity. First, the etiologic factors differ to the human situation: DEN and AAF do not induce the same molecular alterations as do HBV, HCV or alcohol. However, citing Peter Bannasch, a German pathologist, expert in rodent carcinogenesis since the sixties: "*Morphological, microbiochemical and molecular biological approaches in situ have provided evidence for striking similarities in specific changes of the cellular phenotype of preneoplastic FAH emerging in experimental and human hepatocarcinogenesis, irrespective of whether this was elicited by chemicals, hormones, radiation, viruses or, in animal models, by transgenic oncogenes or Helicobacter hepaticus."* (240). The second model we used recapitulates the inflammatory and fibrotic environment, similar to that faced in humans. Nevertheless, as the molecular pathways involved with these chemicals are not perfectly known, it is difficult to affirm a parallelism between the animal and human situation. Second, we can never for sure exclude a pharmacologic interaction between the carcinogenic and therapeutic drugs used, although different ways of administration, metabolic pathways (CYP involved among other things) and significant delays between administrations are some evidence for the contrary. Third, the lesions of interest, i.e. the FAH in the first model and the dysplatic and HCC nodules in the second model, were difficult to identify macroscopically. The possibility to isolate those

areas for mechanistic analyses was therefore challenging. To overcome this, we used double immunohistochemistry for the first experiments, taking advantage of the sensitivity of GSTp staining to identify FAH. Another approach would have been to use laser microdissection, but the technique was locally still under development, with poorly reproducible results.

VIII. Perspectives:

Among interesting features we observed in our experiments, the effect of lanreotide on fibrosis induced by DEN deserves further analysis, as well as its anti-angiogenic and anti-proliferative properties. The way LAN inhibits fibrosis was not explored in the experiment, because we did not expect to observe it with this magnitude. So, the next steps would be to assess effect of LAN *in vitro* in several models:

- To expose hepatic stellate cells *in vitro* to LAN, as these cells are the main actors in liver fibrogenesis. Indeed, it would be interesting to see if the antifibrotic effect observed in our experiments is acting through an inhibition of the activation of HSC into myofibroblasts. For this, it would be worthwile to repeat the DEN model and isolate HSC at various timepoints to have an idea of their phenotype during the whole carcinogenic process.
- To demonstrate and locate the presence of somatostatin receptors on isolated hepatocytes, liver endothelial cells, Küpffer cells and stellate cells at these timepoints.
- To assess the anti-angiogenic effect of LAN, we should evaluate the production of the main growth factors involved, including VEGFA (241) but also other VEGF isoforms and receptors, by neoplastic cells, endothelial cells.
- To understand the molecular pathways by which lanreotide decreases hepatocellular proliferation. Explore in more details the actors of the cell cycle, and of known important gate-keepers involved in liver carcinogenesis: p53, p16, and their inhibitors, such as gankyrin and Mdm2, the main inhibiting regulators responsible for p53 ubiquitination and further degradation by the proteasome. These pathways should be explored on neoplastic cells, but also on surrounding cells, as it has been shown that recurrence of HCC after resection is dependant

much more on genetic signature from the background tissue than from the initial tumor itself (47).

- Finally, apoptosis, which was induced upon LAN treatment when injected to the animals from the beginning of the experiments, seems to play a role in the anti-cancer effect of LAN. So, the apoptotic pathways involved should be analysed, again separately in surrounding liver as well as in HCC.

In the future, lanreotide administration deserves to be evaluated in humans, for example after the curative treatment of a first HCC, in tertiary prevention.

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