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AN HISTOPATHOLOGICAL AND STEREOLOGICAL STUDY OF PROLIFERATIVE HEPATOCELLULAR LESIONS IN A FISH MODEL OF EXPERIMENTAL CARCINOGENESIS AND IN A SENTINEL FISH

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This thesis is especially dedicated to the memory of my father, from whom I learned that life goals should be accomplished with work and modesty and at the end I should always remain true to myself. I really miss him...

CONTENTS

RESUMO

O cancro do fígado é uma doença grave presente em todo o mundo e transversal a várias espécies. Os peixes têm sido usados como modelos de hepatocarcinogénese alternativos aos roedores, para além da sua utilização como sentinelas ambientais, para detectar a presença e os efeitos de xenobióticos nos sistemas aquáticos. Os tipos de lesões e o padrão de desenvolvimento das neoplasias hepatocelulares são semelhantes em peixes e em mamíferos. Nestes últimos, as lesões com origem hepatocelular estão representadas pelos focos de alteração celular (FCA) considerados como lesões pré-neoplásicas, tumores benignos e malignos. No entanto, a falta de concordância entre autores na classificação das lesões e a dificuldade em diferenciar certas categorias lesionais são realidades actuais que têm dificultado a análise comparativa dos estudos existentes. Neste sentido, a uniformização dos critérios de classificação é crucial e é esse o principal objectivo deste estudo, nomeadamente verificando se uma avaliação quantitativa com parâmetros estereológicos é útil para discriminar lesões num sistema de classificação**.**

Foram usados dois parâmetros estereológicos: o volume relativo do núcleo e o volume nuclear médio pesado pelo volume dos hepatócitos com o objectivo de estudar lesões nodulares com origem hepatocelular num espécie de peixe usada como modelo de hepatocarcinogénese (truta-fário, *Salmo trutta*) e numa espécie de peixe-chato (solha-escura-do-mar-do-norte, *Limanda limanda*) considerada sentinela ambiental. Essas determinações foram também realizadas nas áreas de parênquima hepático morfologicamente normal de trutas usadas no protocolo de hepatocarcinogénese (expostas a iniciador, expostas a iniciador mais promotor e não-expostas). O estudo foi feito em microscopia óptica, usando amostras de fígado processadas em parafina e um equipamento de análise estereológica — CAST-Grid (Olympus, Denmark).

De acordo com os critérios usados, todas as lesões observadas na truta foram diagnosticadas como FCA, enquanto na solha-escura do-mar-do-norte existiam também lesões neoplásicas. O diagnóstico de neoplasia foi feito apenas quando se observava compressão do parênquima adjacente associada a atipia celular e arquitectural. As duas estimativas estereológicas eram significativamente diferentes nas lesões e no parênquima normal, para além de que se terem revelado importantes na resolução de casos com aspectos histológicos duvidosos. No que respeita à truta, as características morfológicas e as estimativas permitiram identificar o FCA anfofílico como um subtipo particular. Adicionalmente, os valores do volume relativo do núcleo e do volume nuclear médio pesado pelo volume dos hepatócitos eram diferentes nos fígados dos animais expostos apenas ao iniciador, ao iniciador e ao promotor e não-expostos. No caso da solha-escura-

do-mar-do-norte, as estimativas estereológicas não permitiram, dentro dos limites do estudo, diferenciar as lesões neoplásicas dos focos, contudo o número de lesões neoplásicas era baixo e será necessário um maior número de neoplasias para estabelecer a eventual utilidade destas ferramentas estereológicas na distinção de lesões pré-neoplásicas de neoplásicas.

Desta forma, conclui-se que os parâmetros esterológicos testados são úteis na avaliação quantitativa dos efeitos estruturais associados ao processo de hepatocarcinogénese nos peixes; adicionalmente, estes parâmetros poderão ser relevantes para o diagnóstico e determinação do grau histológico de lesões hepatocelulares induzidas e espontâneas. Prevemos também a sua potencial utilidade em estudos de dose-efeito.

ABSTRACT

Liver cancer is a serious disease present worldwide and across species. For long, fishes have been used not only as alternative models to rodents in hepatocarcinogenesis studies, but also as important environmental sentinels, testifying the presence and effects of xenobiotics in aquatic systems. The lesions and patterns of neoplastic development are interestingly similar in the fish and mammals hepatocarcinogenesis. In fishes, the lesions with hepatocellular origin are represented by the foci of cellular alteration (FCA) — considered to be preneoplastic, benign and malignant tumors. However, the lack of consistency in classification among authors, added to the difficulty in differentiating between categories of proliferative lesions, is nowadays a reality that jeopardizes comparative analyses of studies. In this vein, standardization of criteria is demanding and contributing to this effort was the main goal of our work, namely by assessing whether a quantitative evaluation would be helpful for discriminating the cases in a histopathological classification system.

Two stereological parameters were used: the fractional volume and the volume weighted mean nuclear volume of the hepatocyte nucleus, in order to study the hepatocellular nodular lesions in a fish model of hepatocarcinogenesis (brown trout, *Salmo trutta)* and in a feral sentinel fish from the North Sea (dab, *Limanda limanda*). We also determined those values in healthy-looking hepatocytes of brown trout livers derived from the hepatocarcinogenesis protocol (exposure to the initiator only, to the initiator followed by a promoter and non-exposed). The study was made at light microscopy using routine paraffin sections of livers sampled and the stereological workstation CAST-Grid (Olympus, Denmark).

According to our selected criteria, all the lesions in brown trout were FCA, whereas in dab neoplastic lesions were also present. The diagnosis of neoplasia was done only when compression of surrounding parenchyma co-existed with cellular and architectural atypia. Estimates of both stereological parameters were significantly different between the lesions and the normal surrounding parenchyma, and were important to solve cases with doubtful histopathological features. Regarding the trout, we were able to recognize the amphophilic subtype of FCA as a distinctive lesion, both qualitatively and quantitatively. Besides this, in the normal parenchyma the two parameters also differed among livers of the three groups (non-exposed, initiator only and initiator plus promoter exposed trout). In the dab, the estimates could not satisfactorily differentiate the neoplastic lesions from the FCA; but, as the number of neoplastic lesions was low, we hypothesize that a larger number of

neoplastic cases would allow a full establishment of the utility of the stereological tools for discriminating neoplastic and preneoplastic hepatocellular lesions.

We concluded that the tested stereological parameters are useful in the evaluation of structural effects associated with hepatocarcinogenesis in fish; additionally they may be valuable in the diagnosis and grading of induced and naturally-occurring hepatocellular lesions. We also see some potential for dose-effect studies.

INTRODUCTION

Historical overview of chemical carcinogenesis and genetic origin of cancer

Cancer is an anciently known disease, being the first description attributed to Hippocrates (460-377 A.C.) and the term “neoplasia” credited to Galeno, in the II Century, referring to the growth of a body area adverse to nature (Oliveira *et al*. 2007). The word neoplasm derives from the Greek — *neo* (=new) and *plassein (*=to form or thing formed), meaning the autonomous growth of tissues that escaped normal cellular controls and exhibits variable degrees of similarity to their precursors (Giordano *et al*. 2008). In the XVIII Century, Pott and Hill described cancerous alterations in the skin of the scrotum and in the nasal mucosa in a few patients, and for the first time related those to long-time environmental exposure to soot and snuff, respectively (Luch 2005). In the XIX Century, a high incidence of bladder cancer was observed in chemical and rubber industrial workers in Europe (Oliveira *et al*. 2007). At this point, the scientific community recognized that cancer could develop after environmental or occupational exposure to chemical compounds. The next imperative steps were the systematic inquiry and replication of the disease in experimental systems, and this was achieved with the induction of cancer in laboratory animals (Luch 2005). The first experimental study in chemical carcinogenesis was carried out in 1915 (Yamagiwa and Itchikawa 1918). These pathologists demonstrated for the first time that multiple topical applications of coal tar on the animal skin produced skin tumours, thus substantiating the Pott’s observation that chimney sweepers had an increased incidence of cutaneous scrotal cancer (Smart 2004). In fact, Yamagiwa and Itchikawa are considered the founders of experimental chemical carcinogenesis. Their pioneer work is a milestone in the relation between human epidemiology studies and animal carcinogenicity, marking the transition to a modern era of experimental cancer research (Smart 2004; Luch 2005). By that time, it was recognized that chemicals may cause cancer, but the identification of individual molecules and the knowledge of their mechanisms, at cellular level, were still lacking (Loeb and Harris 2008). Chemists at the Royal Cancer Hospital in London embraced this challenge and later on isolated a single active carcinogen from coal tar, named as benzopryene, a polycyclic aromatic hydrocarbon (Smart 2004). Simultaneously, studies using animal models identified aromatic amines as carcinogenic, adding further data on the carcinogenic properties of industry chemicals (Luch 2005).

In the first half of the XX Century, it was believed that cancer was caused by interactions between chemicals and proteins in specific tissues (Luch 2005). The understanding of the

carcinogenesis process at the cellular level was only possible after the discovery of DNA as the genetic material and its structural description by Watson and Crick (1953). Afterwards, it was realized that DNA represented the target of carcinogens, and that mutations were the keys of underlying the process (Loeb and Harris 2008).

Epidemiological and animal carcinogenicity studies have provided sufficient evidence that exposure to a variety of synthetic chemicals and naturally occurring agents are associated with human cancer (Smart 2004). In fact, nowadays it is easy to find a long list of chemicals that had been implicated in tumour formation (Minamoto *et al*. 1999). For example, exposition to amine dyes is considered as a risk factor to the development of bladder cancer, whereas the association between cigarette smoking and lung cancer is well acknowledged (Smart 2004).

The myriad of studies conducted in chemical carcinogenesis allowed understanding of the neoplastic transformation of the cell as a multistep process that can be divided in three distinct stages: initiation, promotion and progression (Giordano *et al*. 2008). The initiation corresponds to a fast event of DNA damage in a cell, which, in the absence of DNA repair mechanisms, tends to the immortalization — *i.e.,* successive division and transmission of the DNA mutation to daughter cells (Oliveira *et al*. 2007). The next stage, promotion, is the process by which the initiated cell clonally expands into a visible mass — the selective growth advantage effect (Barrett 1993; Loeb and Harris 2008). The most important activity of promoters is mitogenesis, either by delaying the natural inhibition of the quiescent cells or by diminishing the number of cells in G0 phase (Oliveira *et al*. 2007). This means that the effectiveness of a promoter depends on the physiological state of the target tissue; for instance, polycyclic hydrocarbons are highly carcinogenic in neonatal mice, but the same exposure in adults is noncarcinogenic, due to the lack of cell proliferation in the organ at that age (Barrett 1993). Promoters may not interact directly with the DNA, but at least they must produce epigenetic effects — *i.e.,* cellular events related to proliferation, differentiation, or apoptosis, without altering the nucleotide sequence of DNA (Hanahan and Weinberg 2000; Smart 2004). The promoter effect is reversible, meaning that regression of cell proliferation can occur after the removal of the promoter (Oliveira *et al*. 2007). The final histological picture of the promotion phase is named as preneoplastic lesion (focus) or benign tumour (Barret 1993). The cells in these lesions must undergo additional genetic and/or epigenetic changes in order to acquire a malignant biological behaviour (Oliveira *et al*. 2007). This is the progression, the last stage of the multistep model. At the end of it, the cell presents the six hallmarks of a cancerous cell:1) self-sufficiency in growth signals; 2) insensitivity to antigrowth signals; 3) ability to evade apoptosis; 4) limitless replication potential; 5) sustained angiogenesis; 6) capacity to

invade tissues and metastasize (Hanahan and Weinberg 2000; Modiano and Breen 2007). Several lines of evidence showed that the transformation of a normal cell to a cancer requires multiple heritable changes. Major evidence came from the work of pathologists and the observation of multiple stages of tumour progression, such as dysplasia, foci of altered cells and carcinoma *in situ* in different organs (Barrett 1993). Even if this three-stage model of carcinogenesis is nowadays considered an oversimplification, it is still adequate from an operational and academic point of views (Barrett 1993; Oliveira *et al*. 2007).

The evidence for the genetic origin of cancer is now irrefutable; however, the exact number and/or types of genes involved in the neoplastic transformation remains unknown in many neoplasms (Barrett 1993; Modiano and Breen 2007). Three classes of genes are considered the main targets of mutations in neoplastic development: 1) proto-oncogenes; 2) tumor suppressor genes; 3) DNA repair genes (Yeo 1999). The proteins resulting from the proto-oncogenes participate in the cellular signalling transduction pathways, being involved in the regulation of normal cell growth, differentiation or apoptosis (Smart 2004). Proto-oncogenes altered by a mutation (independently of the type) are named oncogenes, which are responsible for positive proliferative signs to cells (Barrett 1993). This kind of mutation is known as dominant “gain-of function”, meaning that the activated allele dominates over the wild type allele and, consequently, the phenotypic change is achieved (Argyle and Khanna 2007). On the other hand, tumor suppressor genes encode proteins that generally act as negative regulators of cell proliferation or regulators of cell death (Smart 2004). These genes were discovered in studies of inherited cancer syndromes in humans — namely the retinoblastoma of children (Argyle and Khanna 2007). When tumor suppressor genes are inactivated by allelic loss, point mutations, or chromosome deletion, they are no longer able to control the cellular growth (Smart 2004). This effect is only achieved when both alleles are inactivated — the so-called loss-of-function defect that represents the rational basis for the “two hit” theory of tumorigenesis proposed by Knudson (Argyle and Khanna 2007). Finally, the last class of genes deals with DNA repair, enabling the cell to maintain their genomic fidelity (Oliveira *et al*. 2007). These may be also affected by loss-of-function mutations, producing the mutator phenotype in the cells, *i.e.*, accelerating the accumulation of random mutations, including those in oncogenes and tumor suppressor genes (Sarasin 2003; Beckman and Loeb 2006). Even the mutation of DNA repair genes is not by itself oncogenic, it would contributed indirectly to the acceleration of the carcinogenesis process (Beckman and Loeb 2006).

Human liver cancer

More than 100 different diseases are collectively termed cancer, but each individual type has distinct biological and clinical features (Fearon 1999). Liver cancer comprises different histological primary hepatic neoplasms that include hepatocellular, biliary, and vascular tumors (Hamilton and Aaltonen 2000). Among these, the hepatocellular carcinoma (HCC) is the most common, representing more than 80% of the cases (Farazi and Depinho 2006).

HCC affects all world populations, although countries differ in incidence rates — reflecting the regional differences in the presence of specific etiological factors (Farazi and DePinho 2006). The incidence of HCC is particularly high in some African and Asiatic countries, even though recent studies documented a marked increase in the number of cases in Western Europe and United States (Philip *et al*. 2005). According to the International Agency for Research on Cancer of the World Health Organization, in 2008 the liver cancer has become the fifth most common cancer in men and the seventh in women, and most of the burden was in developing countries, where almost 85% of the cases occur. There were an estimated 695 000 deaths from liver cancer in 2008 (478 000 in men, 217 000 in women), and because of its high fatality, liver cancer is the third most common cause of death from cancer worldwide (see reference — Globocan 2008).

The marked geographic variation of the incidence of HCC prompted the investigation of location-specific etiological factors (Leong and Leong 2005; Farazi and DePinho 2006). These factors could be divided into biological, environmental and genetic, and included infection by hepatitis B and C virus, ingestion of aflatoxin-B1 contaminated food, chronic alcohol consumption and other cirrhosis-inducing conditions (*e.g.,* hereditary hemochromatosis and α-1-antitrypsin deficiency). Nowadays, it is widely accepted that HCC is unlikely to be due to a single cause and that liver carcinogenesis involves interplay of etiological and host factors (Gomaa *et al*. 2008). The majority of HCC arise in the setting of chronic hepatitis and cirrhosis (Leong and Leong 2005). The end-stage of cirrhosis is actually the major risk factor for development of the tumor in western countries — over 90% of cases of HCC occurred in the background of liver cirrhosis (Gomaa *et al*. 2008); contrasting to this, in Asia and Africa most cases developed in non-cirrhotic livers (Blum and Spangenberg 2007).

Regarding the pathogenesis of HCC, it is currently accepted that the state of chronic inflammation with prolonged hepatocyte injury and consecutive proliferative and regenerative responses provides the mitogenic and mutagenic environment to precipitate secondary genetic events, resulting in unrestrained growth and malignant transformation

of hepatocytes (Leong and Leong 2005; Blum and Spangenberg 2007; Newell *et al*. 2008). Inflammation, continuous rounds of necrosis and regeneration, and oxidative stress are characteristic of hepatitis B and C virus, as well as alcohol induced hepatocarcinogenesis (Farazi and DePinho 2006). Hepatitis B virus (HBV) has DNA and is endemic in sub-Saharan Africa and some Asiatic countries, like China (Newell *et al*. 2008). It causes acute and latent hepatitis and during prolonged infection, the viral DNA is able to integrate the host cell genome. Such an event can result in the activation of oncogenes, inactivation of tumor suppressor genes or transcriptional transactivation of mitogenic factors (alteration in cancer-related genes), thus contributing to neoplastic transformation of infected hepatocytes (Leong and Leong 2005). HBV also encodes a variety of structural proteins, like the protein x (HBx) (Farazi and DePinho 2006). The inappropriate production of this protein has been shown to be sufficient for the malignant transformation (Newell *et al*. 2008). In fact, the contribution of HBx to HCC seems to be mediated by activation of protein kinases pathways and inactivation of p53 (Leong and Leong 2005). HBV vaccination began in the early 1980s, leading to a decline of the rate of HCC in recent years: this was observed in countries like Taiwan and China, especially in cases of children and adolescents (Ni *et al*. 2001).

Hepatitis C virus (HCV) is now emerging as a risk factor for HCC in western countries: epidemiological studies have shown that up to 70% of patients with HCC have anti-HCV antibodies in serum (Montalto *et al*. 2002). In contrast with HBV, the HCV is a RNA virus that does not integrate the host genome (Leong and Leong 2005). Their relation with HCC seems indirect, by causing chronic hepatic disease, with a lag time of 20-30 years between the infection and the development of malignancy (Hamilton and Aaltonen 2000).

Another factor leading to HCC is aflatoxin B1, which is produced by a fungus (*Aspergillus* spp.), being an occasional contaminant of food (*e.g.,* grain, corn, peanuts) in countries with humid climatic conditions. Dietary ingestion of the toxin leads to an increased risk for the development of HCC (Farazi and DePinho 2006), which is potentiated if there is a coexisting HBV chronic infection (Hamilton and Aaltonen 2000). It has been suggested that the fungal toxin can induce mutations in crucial regulatory genes, like p53, thus leading to HCC (Gomaa *et al*. 2008).

Finally, the alcohol represents the leading cause of chronic liver disease, being the most important risk factor in western populations (Farazi and DePinho 2006). Patients with alcohol abuse and with coexisting liver disease from other causes (such as chronic viral infection), have the highest risk for tumor development (Hamilton and Aaltonen 2000). Chronic alcohol intake induces a cascade of events that include: 1) increased serum concentrations of proinflammatory cytokines and endotoxin; 2) Kupffer cell activation and

consequent release of cytotoxic chemokines; 3) oxidative stress in hepatocytes (Farazi and DePinho 2006). Typically, alcohol effects culminate in micronodular cirrhosis (Okuda 2007), which is a permissive microenvironment for HCC (Farazi and DePinho 2006).

It should be noted that HCC is preceded in humans by the development of premalignant lesions including dysplastic nodules, adenomatous hyperplastic nodules with hepatocytic atypia and foci of altered hepatocytes (Su *et al*. 1997; Newell *et al*. 2008). Regarding the liver dysplasia, the last convincing evidence suggested that the small-cell subtype rather than the large-cell change should be considered a precancerous lesion (Newell *et al*. 2008). Similar lesions to the foci of altered hepatocytes founded in several animal models of hepatocarcinogenesis (Bannasch 1986) had been described in association with viral and alcoholic induced cirrhosis in humans, being suggested that they should also be considered preneoplastic (Su *et al*.1997; Su and Bannasch 2003).

Fish as a model of hepatocarcinogenesis

The interest in the use of fish species for cancer research arose from the pioneering work of Stanton, who, in 1965, demonstrated the hepatocarcinogenicity effect of N-diethylnitrosamine in the zebrafish, *Danio rerio* (Bailey *et al*. 1996). By that time, the expansion of synthetic chemicals, namely polycyclic aromatic hydrocarbons produced from industries coincided with a number of neoplastic epizootics in fish (Bunton 1996). The data compiled by several investigators in the field were used as a backbone for the development of experimental carcinogenesis protocols. Since then, fish have proven to be useful not only as environmental sentinels, but also as versatile test animals in carcinogenicity bioassays — being considered as an alternative and parallel model to rodents (Bunton 1996; Law 2001). The use of fish models in experimental carcinogenesis has several benefits that include: 1) the low background incidence of neoplasia in fishes; 2) the low cost of the studies; 3) the rapid tumour formation; 4) the ease of exposure to chemicals (Hendricks *et al.* 1994; Bailey *et al.* 1996; Liu *et al.* 2003). The fish liver has been focused in the majority of the field monitoring studies and laboratory protocols dealing with carcinogenesis. In fact, the patterns of hepatic neoplastic development are interestingly similar in the fish and rodent (Bunton 1996). In both, the liver carcinogenesis is a multistep process involving the same stages: initiation, promotion and progression (Okihiro and Hinton 1999). Despite this similarity, some differences exist between fish and mammals respecting the basic liver histomorphology (Okihiro and Hinton 1999). In addition to the absence of lobes, lobules or acini (the portal triads, as defined/located in mammals, do not exist in fish), the fish liver is not arranged into a simple one- to two-cell-thick plate

structure of hepatocytes (typical of superior vertebrates), but in thicker trabeculae and/or tubules having an axis of biliary passages (canaliculus, pre-ductule or ductule) (for a baseline review see Rocha and Monteiro 1999, and for the latest developments see Hardman *et al*. 2007). In fact, 3D reconstructions suggest a dual-layered plate-like *muralium* predominates in larval and later life stages of medaka, *Oryzias latipes* (a common reference model fish), whereas tubule-like formations were saw in the embryonic liver. Another contrasting feature is the variability: fish liver varies markedly due to gender, maturity, diet and season (Rocha and Monteiro 1999).

The fish species used in hepatocarcinogenesis assays differ throughout the world. The rainbow trout (*Oncorhynchus mykiss*) has an important niche in the history of experimental carcinogenesis in fishes (Bailey *et al*. 1996). A related species is the brown trout (*Salmo trutta fario* Linnaeus, 1785), a freshwater fish which is native in many European countries, including Portugal. In the past, the rainbow trout has been established as an indicator of the potential human exposure to carcinogens in the water column or in the aquatic food chain (Bailey at al. 1996). Our research group has already performed an experience with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) exposure to juvenile brown trout for eight months, in which at least preneoplastic hepatic lesions were observed in more than 90% of the fishes (Rocha *et al*. 2006). The already above mentioned medaka, a small freshwater fish native in Asia and Japan, has also been proven to be a useful test organism in the field of oncology; diethylnitrosamine induced hepatic neoplasms have been extensively characterized by microscopy and other morphological and enzymatic methods (Boorman *et al.* 1997; Brown-Peterson *et al*. 1999; Okihiro and Hinton 1999). In seawaters, the flatfishes common dab (*Limanda limanda*) and flounder (*Platichthys flesus*) are the main target species for monitoring purposes in the North Sea and adjacent areas (including the Baltic Sea), according to the guidelines of the International Council for the Exploration of the Sea (ICES) (Feist *et al*. 2004). Such fishes have been used in the main monitoring programme of the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), homed in Weymouth, United Kingdom (UK). In North American monitoring programmes, another flatfish species, the English sole (*Pleuronectes vetulus*), has been widely used to study the effects of contaminants and their propensity to develop liver lesions (Myers *et al*. 1991; Feist *et al*. 2004). The brown bullhead (*Ameiurus nebulosus)* is the key indicator species in the Great Lakes areas (Blazer *et al*. 2006). It is important to note that even in field studies a causal-effect relationship has been established between exposure and carcinogenic processes, such as in the mummichog (*Fundulus heteroclitus*), another environmental fish sentinel, in which a good correlation was saw between the

environmental exposure to polynuclear aromatic hydrocarbons and the development of liver neoplastic lesions (Vogelbein *et al*. 1990; Blazer *et al*. 2006).

Hepatic pathology in fish: neoplasms and related lesions

The proliferative lesions in the liver can have a hepatocellular or biliary origin, being categorized in foci of altered hepatocytes also called foci of cellular alteration (FCA), and in either benign or in malignant neoplasms (Boorman *et al*. 1997; Blazer *et al*. 2006). The FCA were first described in rodents, but have already been described in various feral fish and in experimental fish models of hepatocarcinogenesis (Bannasch 1986). Each of these lesions consists in a group of cells presenting alterations in glycogen storage or in distribution and/or number of organelles, like the ribosomes (Bannasch 1986). The FCA are classified according to the distinctive cytoplasmic staining features in respect to the normal hepatic parenchyma (Feist *et al*. 2004; Blazer *et al*. 2006). Clear cell, vacuolated cell, eosinophilic, basophilic, amphophilic and mixed FCA have been described in diverse fish species, but all sharing the following diagnostic features: normal (or quasi normal) tubular architecture and absence or just a slight compression of the surrounding tissue (Boorman *et al*. 1997; Feist *et al*. 2004). All types of foci can be recognized histochemically in rodent and fish livers by a reduction or absence of the cytoplasmic iron and the exclusion of other hepatic elements, namely bile ducts. There is evidence for the progression of the FCA, especially the basophilic subtype, to neoplastic nodules in rodent models (Goldfarb *et al*. 1983; Bannasch 1986, Mayer *et al*. 2003) and in fish species (Hendricks *et al* 1984; Brown-Peterson *et al*. 1999; Blazer *et al*. 2006). Moreover, a strong and also consistent association between the presence of FCA and hepatic neoplasms was detected in fishes from polluted sites and different models, strongly suggesting that the formers, namely the basophilic subtype, should be regarded as preneoplastic lesions (Myers *et al*. 1991; Brown-Peterson *et al*. 1999; Blazer *et al*. 2006). Although it is not fully clear, it has been proposed by Hendricks *et al*. (1984) that the appearance of basophilic cells, even when being part of a basophilic FCA, is a signal of complete neoplastic transformation in the rainbow trout.

The hepatocellular benign neoplasms are discrete lesions like the FCA, showing some level of tubular thickening, architectural disorganization and absent or inconspicuous cellular and nuclear atypia (Feist *et al*. 2004; Blazer *et al*. 2006). The staining properties of the constituent cells are also used in the classification of hepatocellular adenoma (Feist *et al*. 2004); the basophilic subtype being vastly the most frequent (Myers *et al*.1991). The malignant hepatic neoplasms are also classified by several key morphological and cellular

features, generally related with the invasiveness and loss of cell differentiation (Feist *et al*. 2004). When a nodular hepatic lesion presents cells with nuclei significantly more atypical than the surrounding parenchyma, that lesion should be classified as malignant (Feist *et al*. 2004; Blazer *et al*. 2006). Nevertheless, some authors argued that the differentiation of a large benign hepatocellular tumor from a carcinoma is sometimes speculative if only based on the histological detail (Hendricks *et al*. 1984; Boorman *et al*. 1997). In rainbow trout model, the histological appearance of the tumors has been considered unpredictable for the biological behaviour of the neoplasia, *i.e.*, the majority of the benign looking tumours exhibit malignant activity, namely invasion capacity (Hendricks *et al*. 1984). It has been stressed that there is a lack of consistency in the classification of liver nodular lesions (Boorman *et al*. 1997; Blazer *et al*. 2006) and that standardization in the diagnostic criteria (with other modern methodological approaches besides histopathology) must be accomplished by ichthyopathologists (Boorman *et al*. 1997; Stentiford *et al*. 2005).

Stereology in hepatic pathology

In fish, as in mammals, the evaluation of the carcinogenic risk and effects deriving from chemical compounds still depends much on conventional histopathology (Bannasch 1986; Stine *et al*. 2004). This is considered a primary tool for evaluating the presence, type and extension of preneoplastic and neoplastic lesions (Stine *et al*. 2004). However, it is generally accepted that the histopathologic classification of tumors is based on a subjective, experience-dependent judgement of the investigator/pathologist and on qualitative evaluations of morphologic and cytological features observed in two-dimensional sections (Broxup *et al*. 1988; Sørensen 1992; Blazer *et al*. 2006). More recently, the trend in histopathology is moving toward quantifying the disease process with morphometric, stereologic and cytophotometric methods (Sørensen 1992; Weibel *et al*. 2007; Gibbons *et al*. 2009).

The quantitative studies in human liver, namely karyometric and DNA content analyses, begun in the 70s of the XX Century, by Russian and Hungarian authors (Sàfrány *et al*. 1970; Tasca *et al*. 1970). Afterwards, in the 80s and 90s, with the establishing of the rodents as models of hepatocarcinogenesis, several authors started using morphometric and stereological approaches in hepatic preneoplastic and neoplastic lesions (*e.g.*, Pitot *et al*. 1987; Broxup *et al*. 1988; Campbell *et al*. 1982; Schwarz *et al*. 1995). The more important data obtained by these studies were the number, volume and size of FCA, as well as nuclei counts (*e.g.,* hepatocytes nuclei number), being these considered relevant for screening and classifying carcinogens, and for risk estimation (Xu and Pitot 2006).

Broxup *et al*. (1988) used computer assisted morphometry to evaluate some cellular features, as the nuclear/cytoplasmatic ratio and nuclear area, being their main goal the establishment of objective parameters for differentiating foci and islands of cellular alteration and neoplastic proliferative hepatocellular lesions. One year later, Jack *et al*. (1989) used the latest (design-based) stereology advances, namely the nucleator method, to quantity and compare the cell volume in foci and in the surrounding normal liver tissue. Those authors also obtained the proportion of mononucleated to binucleated cells and the nuclear volume in foci (Jack *et al*. 1990). Other reported goal for the quantitative analysis of liver carcinogenesis was the assessment of the initiation phase by counting the number of hepatocytes positive to the placental glutathione S-transferase, a useful marker for the preneoplastic lesions in rats (Grasl-Kraupp *et al*. 2000). As far as we know, only a recent study focused the attention on the use of quantitative methods for assessing the hepatic neoplasms and FCA in fishes (Stine *et al*. 2004). The authors used stereology to evaluate the volume, distribution and shape of the hepatic tumoral lesions, and to determine the adequate histological sampling strategy, using the mummichog fish (*Fundulus heteroclitus*) exposed to environmental contaminants (Stine *et al.* 2004).

The nuclear size variation (*i.e.*, nuclear size pleomorphism) is one of the primary features to quantitatively grade malignancy (Sørensen 1992; Kumar *et al*. 2003). With modern stereology it is possible to obtain an unbiased estimation of nuclear size pleomorphism (Gunderson and Jensen 1985; Sørensen 1992; Yörükoglu *et al*. 1998). This pleomorphism may be traduced by the so-called volume weighted mean nuclear volume (nuclear $\overline{v}_v$) that involves sampling of the nuclei in sections in proportion to their volume (Sørensen 1992). The unbiased estimation of the nuclear $\overline{v}_v$ is based on measurements of point sampled linear intercepts in, *e.g.*, isotropic uniform randomly oriented sections, as firstly described by Gundersen and Jensen (1985). Estimates of the nuclear $\overline{v}_v$ have been well correlated with prognosis in several human cancers (Soda *et al*. 1999), like urinary, renal, prostatic, endometrial, breast tumors, and in some melanomas (Binder *et al*. 1992; Ladekarl 1998; Yörükoglu *et al*. 1998; Soda *et al*. 1999; Fujikawa *et al*. 2000). In the breast cancer, it has been emphasized that the nuclear $\overline{v}_v$ provides independent information in grading scheme (Ladekarl 1998). Besides being useful as a predictor of prognosis, the nuclear $\overline{v}_v$ has a diagnostic role in cutaneous tumoral lesions (*e.g.*, nevi and melanoma or keratoacanthoma and squamous cell carcinoma), being a tool for the differential diagnosis between benign and malignant forms (Steiner *et al*. 1994; Binder *et al*. 1992).

As far as we know, the stereological estimations of the nuclear fractional volume and of the nuclear $\bar{v}_v$ were never made in the context of studying hepatic proliferative lesions in any model or in naturally occurring injuries. Thus, the value of this stereological estimation in classifying the FCA and neoplastic lesions in hepatocarcinogenesis models remains to be established.

AIMS

With the above in mind, we wanted to tackle qualitatively and, in particular, quantitatively one basic problem that is evident from the literature: the diverse and not always clear criteria for the identification of the lesions associated with fish hepatocarcinogenesis. Thus, on the one hand, we proposed to critically review and, if possible, refine and improve the currently used criteria with further observations, and, on the other hand, we hypothesized that the use of certain stereological tools on the nucleus of hepatocytes inside the lesions could provide further advances on classification and grading.

Facing the key problem, the objectives of this study were: 1) to classify the proliferative hepatocellular lesions found in each specimen (studying two fish species), using combined histopathological diagnostic criteria from different authors and avoiding the pitfalls in the classification systems; 2) to estimate the volume density (also called fractional volume) of the nucleus and nuclear $\bar{v}_v$ (incorporating the nuclear size variability) using stereological tools, in each classified lesion; 3) to compare the stereological estimations obtained in the normal uninvolved liver parenchyma, FCA and neoplastic lesions (if present); 4) to compare the stereological estimates in the different subtypes of FCA (if present) and to determine whether the estimates corroborate the morphological classification; 5) finally, we aim to determine whether the volume density of the nucleus and the nuclear $\bar{v}_v$ are useful for the classification scheme of those hepatic lesions ─ *i.e*., whether those stereological parameters show potential to support the establishment of cut-offs, in order to differentiate normal, preneoplastic and neoplastic hepatocytes.

This study was divided in two parts, in which the baseline aims and analytical approaches are similar, and the data expected to be complementary, differing in the target fish species and background context. In the first part we studied the liver of the brown trout — in the perspective of a laboratorial model of hepatocarcinogenesis — and in the second part we dealt with the liver of the dab — a species used in major biomonitoring programmes in the UK marine waters (Feist *et al*. 2004; Stentiford *et al*. 2005). With such an approach we wanted to see whether the conclusions would be strong enough to hold in different

scenarios. At this point, it is important to stress that regarding the trout our goals also included the stereological estimation of the nuclear fractional volume and of the nuclear $\overline{v}_v$ in normal appearing hepatocytes, in three groups: 1) animals exposed to an initiator plus a promoter; 2) animals only exposed to the initiator; 3) non-exposed animals (negative controls).

MATERIAL AND METHODS

Material

Part I – Brown trout — a fish model for experimental hepatocarcinogenesis

The analyzed material was derived from a former experience conducted by Prof. Eduardo Rocha (ICBAS), in which eyed-eggs of brown trout were exposed to a aerated water bath of 50 ppm of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 1 hour, and then allowed to hatch, absorb the yolk-sac and swim up to feed. Eight-weeks after the initiator (MNNG bath) and among other studied settings, an experimental and a control group were created by exposing the animals for 8 months to (nominal concentrations): 1) 50 µg/L of 17-β estradiol (to act as a promoter); 2) vehicle control (ethanol saline at 0.001%). A third group of non-initiated/non-promoted negative controls, *i.e.*, fish without exposition to MNNG or to 17-β estradiol but maintained in the same conditions, was also included (to discard the natural occurrence of liver neoplasms). Animals were kept in well-aerated closed circuits, with freshwater being totally renewed every 48 hours, with a reposition of the nominal concentrations. Water quality parameters were regularly checked to confirm that they were within optimal ranges for trout maintenance. At the end of the experimental protocol, all the animals were euthanized with an overdose of anesthetics (ethylene glycol monophenyl ether). At the end of the experiment, the fish presented a mean weight of 19 g (CV = 0.60) that did not vary among groups. Livers were excised and sliced into 3 mm thick slabs, fixed for 24 hour in buffered formaldehyde at 4%. Then, the fragments were routinely processed for paraffin embedding, sectioned (at 4-5 µm) and stained with haematoxylin-eosin. For histopathology and stereology, we randomly picked 30 fish per group.

Part II – Dab — an environmental sentinel fish from the North Sea

Twenty five liver slides from the same number of dab were included in this study. They were kindly supplied by Dr. Stephen Feist, from the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, UK, corresponding to animals captured in the North Sea, according to guidelines established by the International Council for the Exploration of the Sea (ICES). According to Feist *et al*. (2004), the fishes were captured by trawling, using appropriate fishing gear, and after landing the animals were transferred to aerated flow-through seawater tanks. Two sampling strategies were used by CEFAS, in order to monitor the liver lesions in flatfishes from the North Sea: 1) 50 fish per station were submitted to macroscopic examination to detect the presence of liver nodules over 2 mm in diameter and all those nodules were sampled; 2) 30 dab with 20-24 cm in total length were also sampled. In cases where there were no gross hepatic lesions, a 3

mm slice was cut longitudinally through the central axis of the liver. If macroscopic lesions were present, a section through the entire depth of the lesion was added. Afterwards the fragments were routinely processed for paraffin embedding, sectioned (at 4-5 µm) and stained with haematoxylin-eosin. A histopathological study was performed herein in order to enumerate and classify all proliferative lesions. Degenerative and inflammatory lesions were not considered, because they were beyond the scope of this study.

Methods

Histopathological study

The nodular proliferative hepatic lesions from trout and dab were classified according to the criteria presented in Table 1. These criteria were based on descriptions from technical reports and articles (Hendricks *et al*. 1984; Boorman *et al*. 1997; Feist *et al*. 2004, Blazer *et al*. 2006). The major criteria were those that must be present to classify the lesion in a specific type, whereas the minor criteria may or not be present, being considered secondary. Three categories of nodular lesions were considered: FCA, hepatocellular adenoma and hepatocellular carcinoma. The FCA were subclassified in either eosinophilic, basophilic or amphophilic, according to the fine staining qualities of the involved hepatocytes. In order to obviate the differences in the intensity of the stain among slides, the subclassification of the FCA took the normal adjoining parenchyma as a reference for staining. For example, the classification of a FCA as eosinophilic implies that it must be composed of cells with a homogeneous cytoplasm that is more eosinophilic than the cytoplasm of the surrounding hepatocytes. Using a criterion from human and rodent analyses, the amphophilic subtype was considered whenever there was a relatively marked increase in both eosinophilia and basophilia in the cytoplasm of the hepatocytes (Su *et al*. 1997; Mayer *et al*. 2003).

Other premises were also considered, like the size — meaning that the FCA with less than 10-12 cells were not registered (Boorman *et al*. 1997) — and the cell origin, since biliary lesions were neglected. For each lesion a colour plate was made herein, based on digital photographs, in different magnifications, and a complete morphological description of the lesion was established. Since a liver slide often had more than one lesion, this was relevant and usually helped the subsequent identification of a specific lesion in that slide, namely when the stereological analysis was to be performed. Additionally, this allowed the discussion of difficulty on the classification in some lesions.

Table 1 - Diagnostic morphological criteria for proliferative hepatocellular lesions applied herein to brown trout and dab.

Lesion	Major Criteria	Minor Criteria
FCA	No compression of surrounding parenchyma. Normal or exacerbated tubular architecture. Nuclear morphology similar to the parenchyma.	Cells blended into surrounding parenchyma. Rare or absent mitotic figures. Absence of bile ducts and macrophages aggregates.
HC Adenoma	Compression of the surrounding parenchyma. Atypical tissue architecture. Small nuclear and cellular pleomorphism.	Exacerbation of tubular pattern and/or tubular thickening. Increased cellularity. Rarely observed mitotic figures.
HC Carcinoma	Compression and/or invasion of surrounding parenchyma. Atypical tissue architecture (solid pattern). Increased nuclear and cellular pleomorphism (comparing with surrounding parenchyma).	Increased cellularity. Increased mitotic figures. Satellite lesions. Cystic and/or necrotic areas.

Stereological study

All the proliferative hepatocellular lesions identified in the previous part of the work were submitted to a stereological study. For each lesion (FCA or neoplasia) we also sampled a morphologically normal and non-nodular area of liver parenchyma in the same slide, which was taken as an internal control. This was the largest area of remaining normal appearing liver parenchyma; that depended on the number and relative size of the lesions appearing in each slide. Additionally, in the brown trout we performed the same stereological measurements in the normal parenchyma of those animals exposed only to the initiator (MNNG) and to the vehicle, as well as in the negative controls. In all cases, we used a systematic sampling approach in the selection of the fields for the analysis. These were made under the 100x oil immersion objective.

Two stereological approaches were used to estimate the volume density (relative to the liver), or fractional volume, of the nucleus [V_V (hepatocyte nucleus, parenchyma)] and the nuclear $\bar{v}_v$ of the preneoplastic, neoplastic and normal hepatocytes.

With the stereological workstation CAST-Grid (Version 1.5, Olympus, Denmark), we estimated the nuclear $\bar{v}_v$ using the point-sampled intercepts method (PSI) (Gundersen and Jensen 1985; Howard and Reed 1998). This parameter quantifies the nuclear size and pleomorphism, being estimated with a test grid made of parallel lines bearing a systematic pattern of points (Figure 1). Only the nuclear profiles randomly hit by one of these points were sampled. On these profiles, the line segments crossing the point-sampled nucleus were measured from boundary to boundary (Figure 1). Then, the measurements were used to estimate the nuclear $\bar{v}_v$ as:

$$\bar{v}_v(nucleus) = (\pi / 3) \cdot \bar{l}_0^3$$

As to the V_V (hepatocyte nucleus, parenchyma), it was estimated by the point counting method (Figure 2). This is based in a direct proportion of points hitting the object of interest (herein nuclear profiles) and points hitting the reference space. For analytical purposes, this space was the hepatic parenchyma, excluding the vessels that were larger than a sinusoid. The V_V was calculated with the formula (Rocha *et al*. 2001):

$$V_v(nucleus, reference\ space) = \sum P(nucleus) / \kappa \cdot \sum P(reference\ space)$$

in which κ is the ratio between the number of grid points used for targeting the nuclei and those used for the reference space (here in parenchyma). In this study, we designed a test grid in which 9 points were used for the nuclei and 81 points for parenchyma, so that κ = 9.

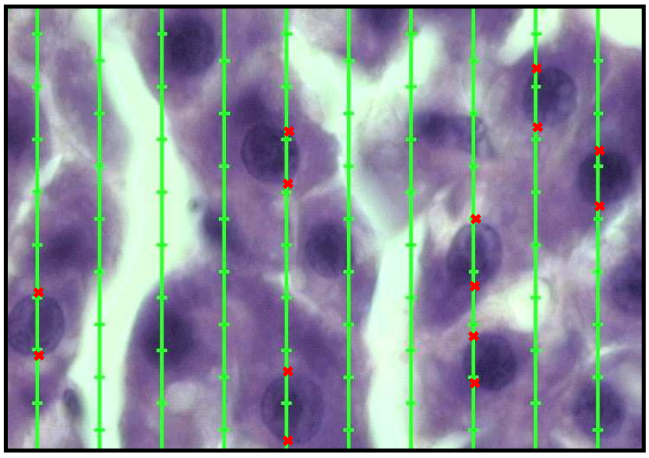

Figure 1 – Estimation of the nuclear $\bar{v}_v$ with the PSI method (see text), in the trout liver. A grid of parallel lines bearing a systematic pattern of points allows a selection of nuclear profiles (in sharp focus, hit by one of the points). In these, the distances between the intersections of the nuclear boundaries with the lines (red crosses) were measured.

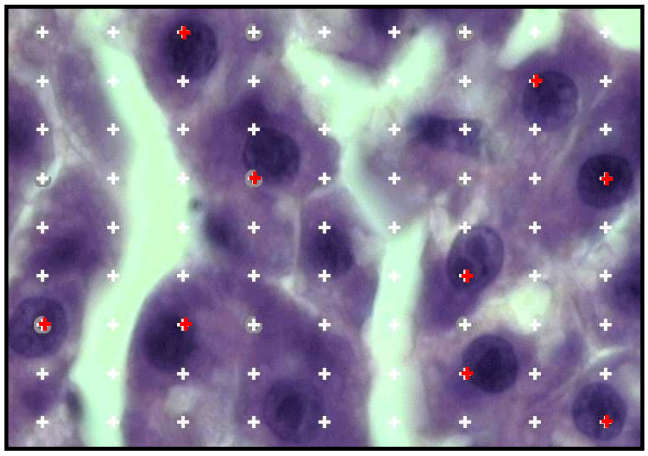

Figure 2 – Estimation of the V_V (hepatocyte nucleus, parenchyma) by the point counting method, in the trout liver. A grid of points is overlaid over the image and the points hitting the nuclear profiles of hepatocytes (red) and the reference space (white + red) are counted. In this study, the reference space excluded vessels larger than a sinusoid.

Regarding the V_V (hepatocyte nucleus, parenchyma) estimation in brown trout, the next mean numbers of points were counted:

- 71 over the parenchyma and 50 over the nuclei in the FCA;
- 110 over the parenchyma and 55 over the nuclei in the normal parenchyma areas of the MNNG plus estradiol group;
- 284 over the parenchyma and 141 over the nuclei in the MNNG exposed livers;
- 408 over the parenchyma and 242 over the nuclei in the negative controls.

Regarding the nuclear $\bar{v}_v$ estimation in brown trout, a mean number of 25, 46, 137 and 164 intercepts of nuclear profiles were measured in each FCA, normal parenchyma area of MNNG plus estradiol exposed animals, normal parenchyma of MNNG exposed animals and of negative controls, respectively.

Regarding the counting for the V_V (hepatocyte nucleus, parenchyma) in dab, the following mean numbers of points were counted:

- 127 over the parenchyma and 78 over the nuclei in the FCA;
- 446 over the parenchyma and 304 over the nuclei in the neoplastic lesions;
- 251 over the parenchyma and 110 over the nuclei in the normal parenchyma areas.

Regarding the nuclear $\bar{v}_v$ estimation in dab, a mean of 27 intercepts of nuclear profiles were measured in each FCA. In neoplastic and normal parenchyma, a mean of 106 intercepts and 41 intercepts were quantified, respectively.

Statistical analysis

All numerical data were subjected to a statistical analysis using the software Statistica (version 9.0) for Windows. The normality and homogeneity of variances were checked before the analysis. In some cases, logarithmic transformation and inversion was made for data normalization before applying the parametric statistical test. When normalization was not achieved, a non-parametric test was chosen. The detection of outliner values was done using the Grubbs´test.

For the normal hepatocytes from negative controls, MNNG and MNNG plus estradiol groups, the data regarding the nuclear $\bar{v}_v$ and V_V (hepatocyte nucleus, parenchyma) were analyzed by one-way ANOVA and by Kruskal-Wallis ANOVA, respectively. The V_V (hepatocyte nucleus, parenchyma) data obtained in the lesions and respective normal surrounding parenchyma (internal control), of MNNG plus estradiol exposed brown trout, were analysed by a t-test for matched paired samples. The data regarding the nuclear $\bar{v}_v$ values of the same lesions and parenchyma areas did not follow a normal distribution; therefore, a Wilcoxon Matched Pairs non-parametric test was used. We also performed a statistical analysis in order to compare the stereological estimations of V_V (hepatocyte nucleus, parenchyma) and nuclear $\bar{v}_v$ in aFCA and in bFCA. The comparison between these two subtypes of FCA was first done after grouping all the cases of each subtype independently of their origin (*i.e.*, independently on their presence in the same liver/animal or in different livers/animals); for this, we used a t-test for independent samples. We also carried out a multiple comparisons test (in this case with the Kruskall-Wallis ANOVA), after grouping all the exclusive bFCA (*i.e.*, bFCA that appeared as the unique subtype in one liver/animal), all the aFCA in the same conditions, and finally all the bFCA or aFCA that co-existed with the respective other subtype. In all occasions, 2-tailed tests were used and results were considered significant for $p < 0.05$.

RESULTS

Histopathological study

Part I – Brown trout

The normal trout liver parenchyma presented hepatocytes organized in tortuous, ramifying plates with at least two-cell thickness. The underlying quite complex 3D-tubular (or partially tubular) structure was masked because the sinusoidal capillaries were either completely or partially collapsed, and also because of its intricate 3D-organization. Elongate to triangular shaped, more euchromatic nuclei were observed in the middle of the hepatocyte plates — those elements were identified as biliary passage lining cells. Associations between blood vessels, arterial and/or venous in origin, with cuboidal to columnar epithelial lining biliary ducts were present. All those elements also appeared isolated; a normal condition in fish.

In MNNG plus estradiol exposed animals, the liver parenchyma in non-focal areas was homogeneous regarding the hepatocytic cytology. Among animals, the hepatocytes presented two morphological variants: 1) moderate to highly vacuolated cells (discrete and non discrete vacuoles) with a small perinuclear basophilic cytoplasmic area and a round to angulated nucleus (Figure 3A); 2) poorly vacuolated hepatocytes with a basophilic uniform cytoplasm and round nucleus (Figure 3B). Regarding the nuclear morphology of the normal appearing hepatocytes, we found moderate anisokaryosis, one to three visible nucleoli, and a coarse to clumped chromatin pattern (*i.e.,* appearing as strands or aggregates) (Figure 4A and B). All the animals in this group presented focal nodular lesions (100% of sample prevalence). A total of 72 FCA were detected and classified as eFCA, aFCA and bFCA (Figure 5); these accounted for 5, 33 and 34 lesions, respectively. All the FCA were discrete lesions, presenting continuity with normal parenchyma and without compression. The cellularity and nuclear morphology was similar (at least qualitatively) to the normal surrounding parenchyma. Despite this, and typically, the cell *muralium* within the FCA was far more outlined from the capillary network than in the normal parenchyma and the nucleoli were more prominent. Mitotic figures were occasionally observed in focal (Figure 5B) and non focal parenchyma. Only in one medium sized lesion we noted compression of the surrounding parenchyma; however, no alteration of the cellular architecture was evident (*i.e.*, no areas of solid arrangement of the hepatocytes were present), allowing the classification as FCA. No clear cell or vacuolated focus was observed in the studied livers.

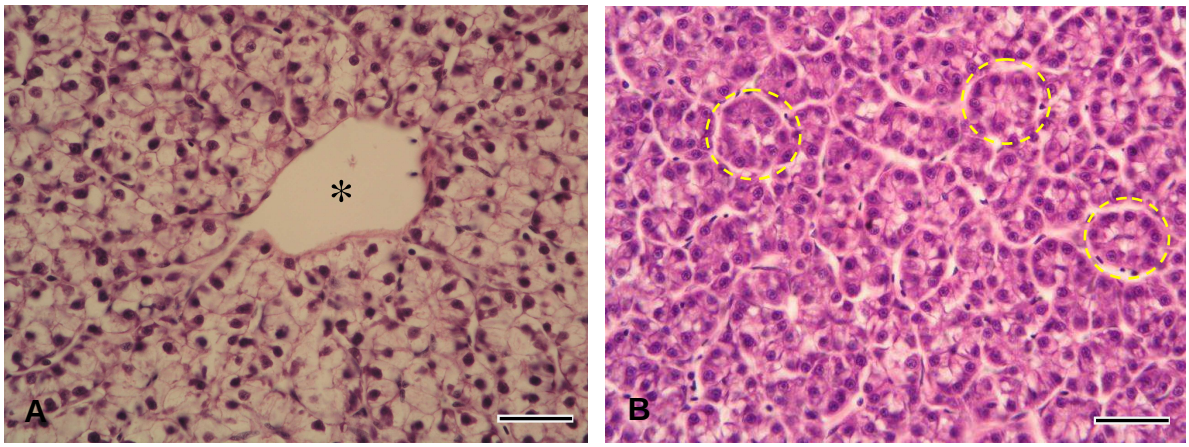

Figure 3 – Normal liver parenchyma in MNNG plus estradiol exposed brown trout. A - Note the highly vacuolated hepatocytes with a very small area of basophilic perinuclear cytoplasmic. The organization of the hepatobiliary architecture is difficult to disclose, not only due to its 3D-complexitiy but also because most of the sinusoids are collapsed. A vein branch is also presented (asterisk). B - Note the poorly vacuolated hepatocytes (discrete vacuoles) with a basophilic cytoplasm. The hepatocellular arrangement and sinusoids with endothelial cell lining are more evident in this specimen, with some tubules standing out (circles). Haematoxylin-eosin (H&E), Bar = 40 µm in A and B.

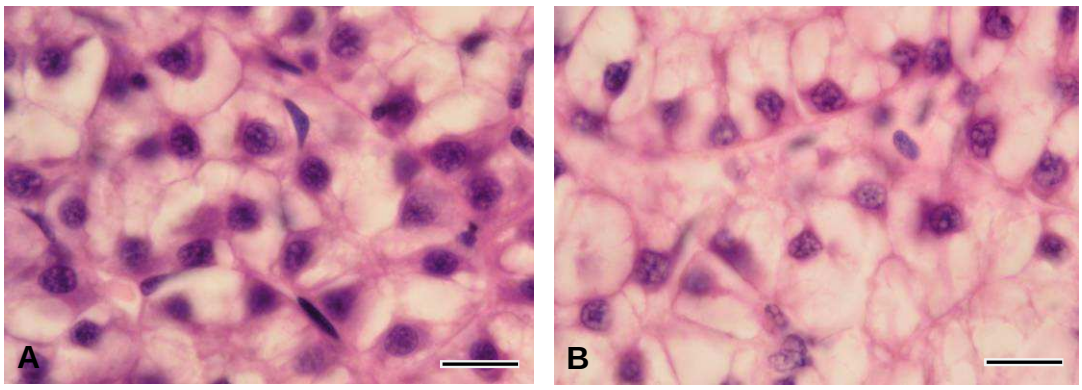

Figure 4 – Nuclear morphology of normal appearing hepatocytes in MNNG plus estradiol exposed brown trout. Note the round to slight angulated nuclear profile, the small visible nucleoli and the strands or aggregates of chromatin. H&E, Bar = 20 µm in A and B.

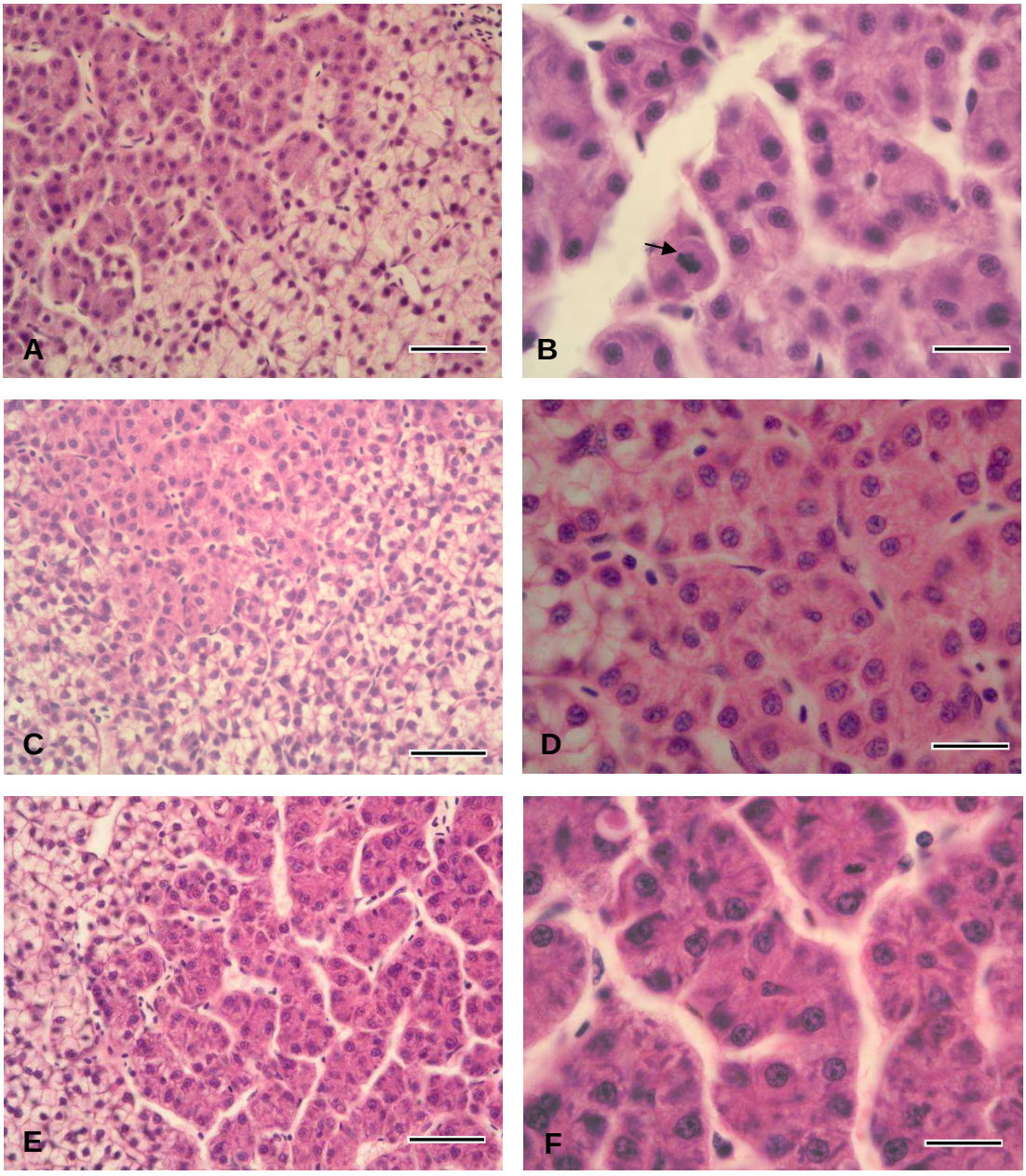

Figure 5 – Examples of the subtypes of FCA in brown trout. The basophilic FCA (A and B) presents hepatocytes with uniformly basophilic cytoplasm (comparing with the surrounding parenchyma), whereas the eosinophilic subtype (C and D) exhibits a uniform cytoplasmic eosinophilia. The amphophilic FCA (E and F) is characterized by a heterogeneous cytoplasm, *i.e.,* with punctuate areas of basophilia and eosinophilia. Note that in all cases the cells of FCA merge directly with the cords of surrounding parenchyma and do not cause compression, but strikingly different staining properties provided an obvious border. Note also a mitotic figure in B (arrow). H&E, Bar = 50 µm (A, C, E); 20 µm (B, D, F).

In trout exposed only to MNNG the liver morphology was similar to the above described, *i.e.,* the two morphological variants described were also observed; the main differences were the increased basophilic areas associated to a decreased (discrete and non-discrete) vacuolization of the hepatocytes cytoplasm (Figure 6A). In non-vacuolated hepatocytes the cytoplasm was basophilic or amphophilic (Figure 6B). The nuclei of hepatocytes showed round profiles, slight anisokaryosis, three small to one central large nucleolus, and also a clumped chromatin pattern with perimembranar condensation (Figure 7A and B). In the 30 livers studied we found 5 with 2 to 3 bFCA, which represented a 17% sample prevalence of animals with lesions.

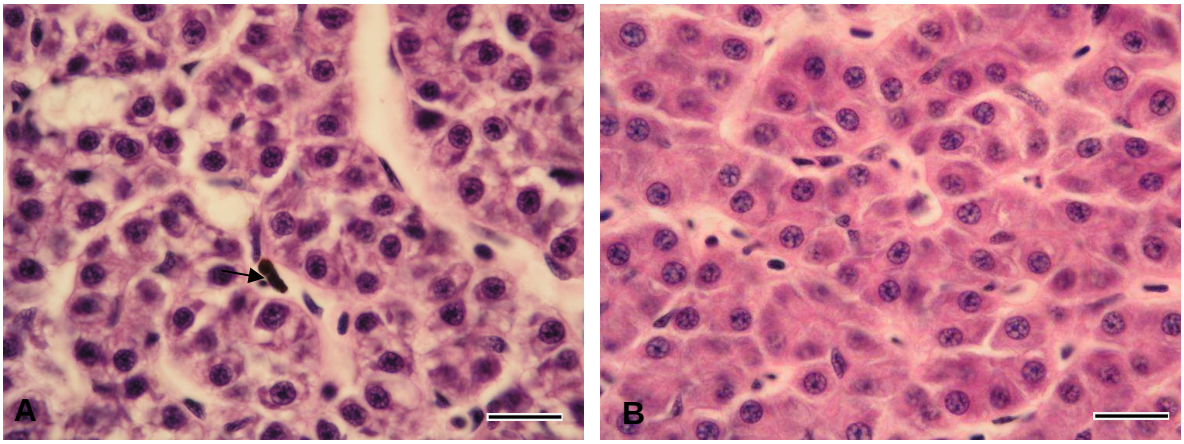

Figure 6 – Normal liver parenchyma in MNNG exposed brown trout. A - Note the moderate vacuolated hepatocytes with basophilic cytoplasmic areas. The sinusoids are distended and a small deposit of melanin is present (arrow). B - Non-vacuolated hepatocytes with amphophilic cytoplasm, round nucleus, one large to three small evident nucleoli and a clumped chromatin pattern are present. H&E, Bar = 25 µm in A and B.

In non-exposed controls no nodular lesions were observed. The degree of cytoplasmic vacuolization of the normal hepatocytes in those animals was low and only occasionally we found non-discrete and discrete vacuoles in the cytoplasm of hepatocytes (Figure 7). In the majority of the non-exposed fish, the hepatocytes presented a homogeneous cytoplasm, either with amphophilic or basophilic tinctorial proprieties. As to the nuclear morphological characteristics, they were similar to those described in the MNNG exposed animals.

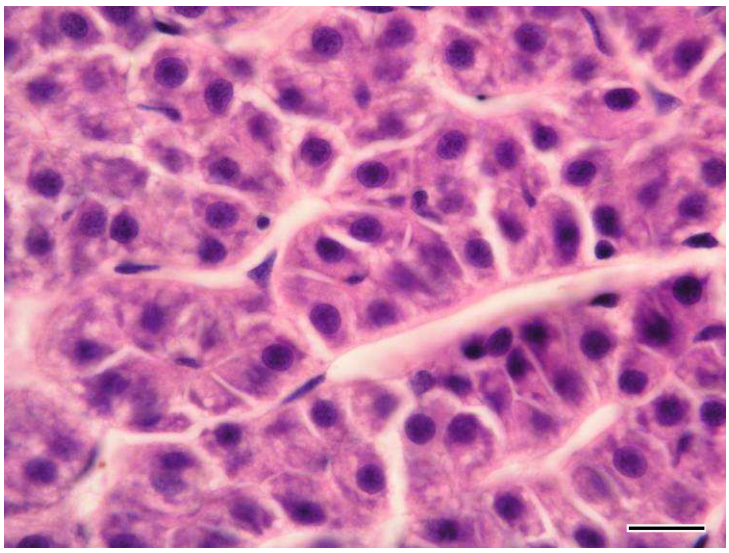

Figure 7 – Normal liver parenchyma in a non-exposed brown trout. Small discrete vacuoles are present in the amphophilic cytoplasm of the hepatocytes. The round nuclei have chromatin clumping. H&E, Bar = 20 µm.

During our evaluation we tried to visually assess eventual differences regarding both the abundance and size of the nuclei of the hepatocytes, as one possible biomarker for cells leaving their normal condition. We detected relatively smaller nuclei in the normal appearing hepatocytes of MNNG plus estradiol exposed animals, comparing with MNNG exposed and negative control animals.

Part II – Dab

The normal hepatocytes of the dab had different degrees of cytoplasmatic vacuolization: 1) completely vacuolated (clear cells without any stained cytoplasmic area) (Figure 8); 2) partially vacuolated (with one macrovacuole or various microvacuoles associated with small basophilic or eosinophilic areas); 3) non-vacuolated (with basophilic or amphophilic cytoplasm) (Figure 9). In those partially vacuolated hepatocytes, a single macrovacuole was more frequent than the presence of various small microvacuoles. The nuclei were generally round and with a central prominent nucleolus; in the vacuolated hepatocytes the nuclei tended to be more heterochromatic, whereas in non-vacuolated cells a reticular chromatin pattern was seen. From the 25 studied animals only 14 displayed hepatocellular nodular lesions. In these we identified 24 nodular lesions: 16 FCA (6 eFCA, 5 aFCA and 5 bFCA), 8 neoplastic lesions (6 adenomas and 2 carcinomas). Six animals presented only neoplastic lesions. The concomitant presence of neoplastic lesions and FCA was observed only once. In livers with more than one FCA, the basophilic and amphophilic subtypes coexisted. No clear cell or vacuolated focus was observed in the studied livers. All FCA were discrete lesions without compression of the surrounding parenchyma. The cellular arrangement and nuclear morphology was similar to the normal surrounding cells. In some FCA the hepatocellular tubules were more evident, but the cellularity did not appear to be increased (at least qualitatively) when comparing to the normal, non-nodular areas.

In all the neoplastic lesions we faced the associations of four features: 1) relative large dimension (*i.e.*, the lesions were at least mesoscopic); 2) compression of surrounding parenchyma (Figure 10A); 3) increased cellularity (with clear tendency to assume a solid arrangement) (Figure 10B, C and D); 4) more prominent nucleoli and anisokaryosis (Figure 10C and D). Additionally, the two cases classified as carcinoma presented distinctive features. The compression was associated with focal irregular borders and small groups of cells tended to invade the adjacent parenchymal tissue (Figure 11A and B). Additionally, areas contained enlarged and thrombotic blood vessels with inflammatory reaction (macrophage-rich) associated to small amount of fibrosis were also observed within the lesion. Only in one lesion the morphology was dubious, since it was middle sized and without compression of the surrounding parenchyma, but it showed an increase of cellularity and more nuclear pleomorphism (anisokaryosis). In the livers without vacuolated normal hepatocytes we did not observe nodular lesions or else noted only FCA. In contrast, in those livers with completely vacuolated hepatocytes we either detected foci and/or neoplastic lesions (specifically, hepatocellular adenomas) or eventually observed no nodular lesions at all. Regarding the two cases of carcinoma, they were present in a background of partially vacuolated normal hepatocytes.

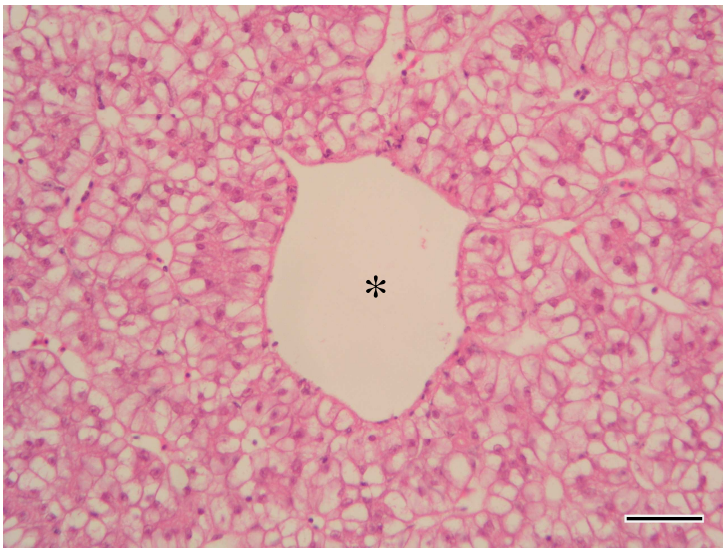

Figure 8 – Normal liver parenchyma in the dab. The marked vacuolization of hepatocytes can be noted as well as the relatively indistinct hepatocellular architecture. The capillary network is squeezed by the cell *muralium* and noted as collapsed sinusoids. A vein branch is evident (asterisk). H&E, Bar = 35 µm.

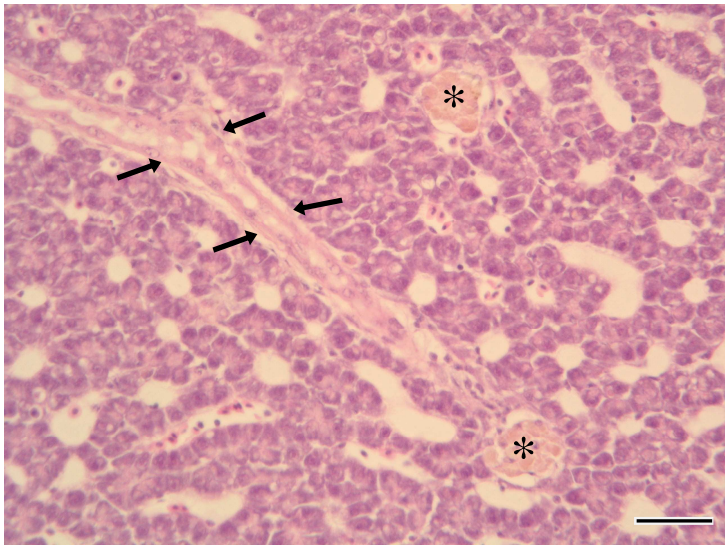

Figure 9 – Normal liver parenchyma in the dab. In this specimen the hepatocytes present stained basophilic cytoplasm and inconspicuous vacuoles. Plate-like and even clear tubular arrays with different cell thickness are herein more evident because sinusoids are dilated. Note the bile duct lined by a simple cuboidal epithelium (arrows). Clusters of pigmented cells, termed “macrophage aggregates” are also present (asterisks). H&E, Bar = 35 µm.

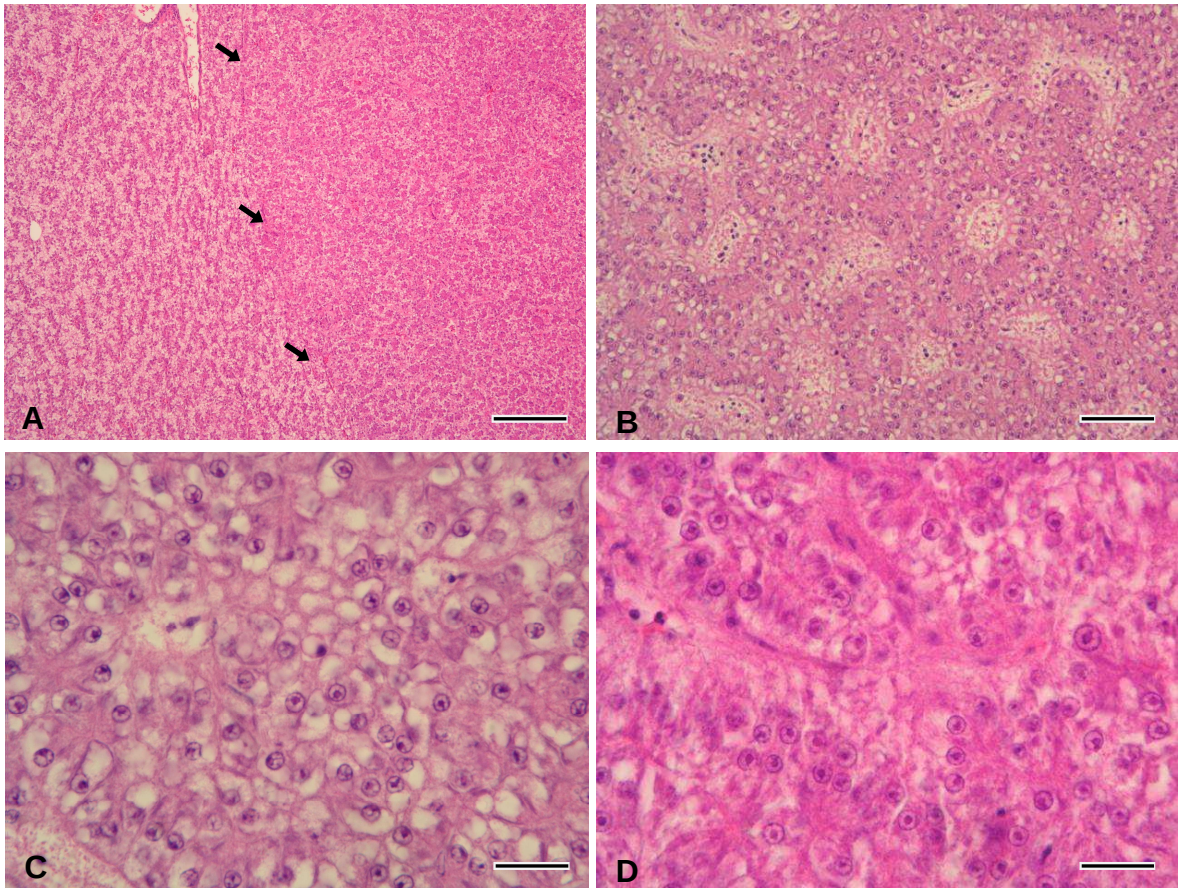

Figure 10 – Neoplastic lesions in the dab. The transition to the neoplasia (arrows) is evident because the surrounding parenchyma appears compressed (A). In the intermediate magnification (B), the space between neighbor sinusoids is thicker as a consequence of the increased hepatocytic cellularity. In the high magnification (C and D), the increased cellularity is obvious, as well as the prominence of the nucleoli and the anisokaryosis (in D). H&E, Bar = 100 µm (A); 50 µm (B); 20 µm (C, D).

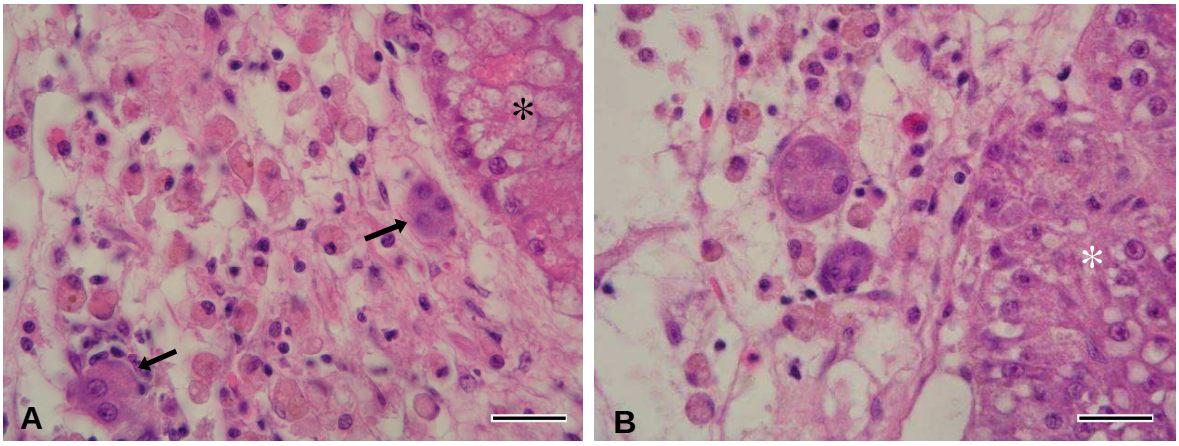

Figure 11 – Features of a malignant hepatocellular neoplasia in the dab. A - Islands of neoplastic hepatocytes (arrows) appeared in the border between the neoplastic tissue and normal parenchyma (asterisk). An inflammatory reaction rich in macrophages, with erythrophagocytosis is seen surrounding the groups of neoplastic cells. B – Analogous features are observed in this image that shows the neoplastic border (white asterisk). H&E, Bar = 20 µm (A, B).

Stereological study

Part I – Brown trout

The 72 FCA diagnosed in the MNNG plus estradiol exposed animals along with their respective normal parenchyma (reference internal controls) area pairs (see Methods) were submitted to stereological estimation of V_V (hepatocyte nucleus, parenchyma) and nuclear $\bar{v}_v$. The same stereological approach was additionally done in 30 MNNG exposed livers and in 30 negative control livers.

The V_V (hepatocyte nucleus, parenchyma) derived for the normal parenchyma varied between groups, being significantly higher ($p < 0.01$) in the negative controls when compared to the MNNG plus estradiol and to MNNG exposed animals (Figure 12). In parallel, the V_V of the hepatocyte nucleus within parenchyma of FCA was significantly ($p < 0.01$) much higher than the V_V estimated in the normal parenchyma (Figure 13).

In opposition to above trends of the V_V (hepatocyte nucleus, parenchyma), the nuclear $\bar{v}_v$ increased from the negative controls to MNNG plus estradiol and to MNNG exposed animals (Figure 14). The differences between all the groups were statistically significant ($p < 0.01$). In the same direction, in FCA the nuclear $\bar{v}_v$ was much higher ($p < 0.01$) in hepatocytes within the lesion than in those in the surrounding parenchyma (Figure 15).

The V_V (hepatocyte nucleus, FCA parenchyma) did not differ between the three FCA subtypes; however, the nuclear $\bar{v}_v$ was significantly higher in aFCA than in bFCA ($p < 0.05$) (Figure 16).This difference was further emphasized when the bFCA and aFCA were compared when considering subgroups of data made of only of those bFCA and aFCA appearing as the exclusive subtype of lesions presented in a liver (Figure 17). Regarding the eFCA, the nuclear $\bar{v}_v$ of their cells did not differ from the values obtained in the cells of the other two subtypes of foci ($p > 0.05$).

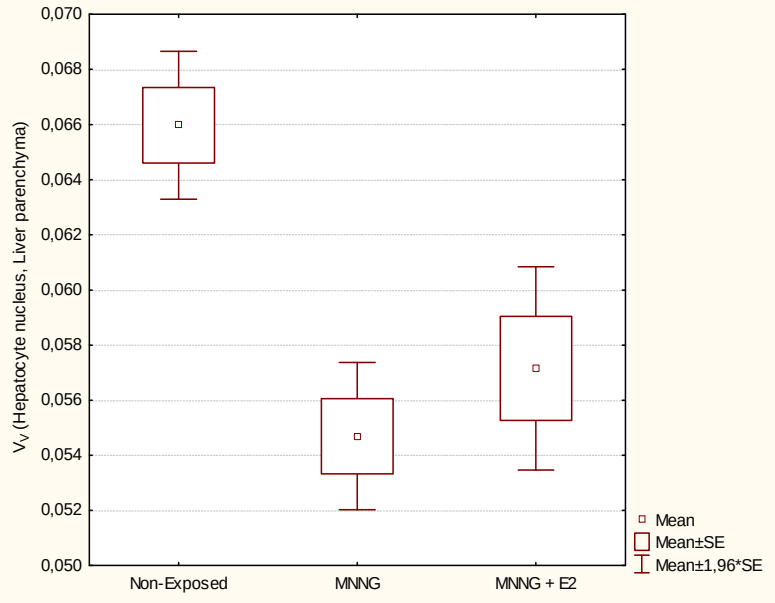

Figure 12 – Brown trout. Graphic plotting of V_V (hepatocyte nucleus, parenchyma), estimated in the normal parenchyma of negative controls (Non-exposed), MNNG, and MNNG plus estradiol (MNNG + E2) exposed brown trout. The two latter groups did not differ, but both diverged from the Non-Exposed one ($p < 0.01$).

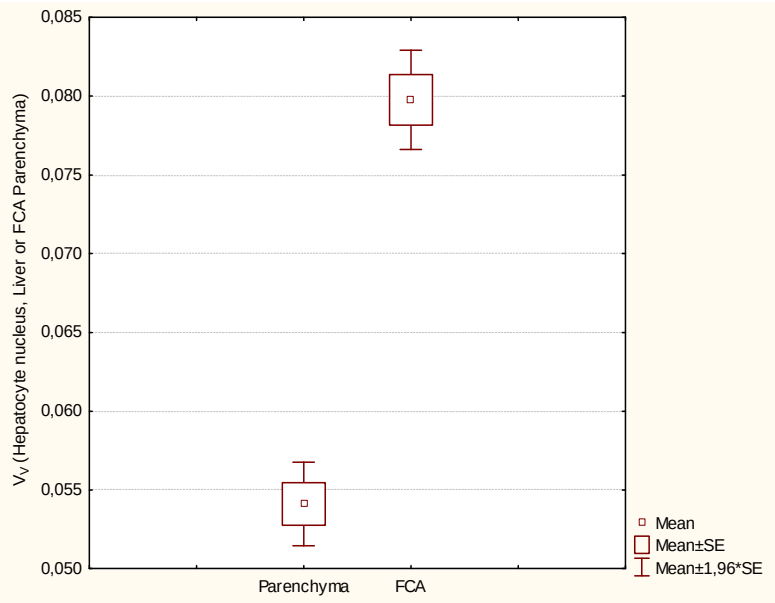

Figure 13 – Brown trout. Graphic plotting of the V_V (hepatocyte nucleus, parenchyma) within the focal parenchyma (FCA) *versus* in the normal liver tissue (Parenchyma). The V_V was significantly higher in FCA than in the surrounding (normal) parenchyma ($p < 0.01$).

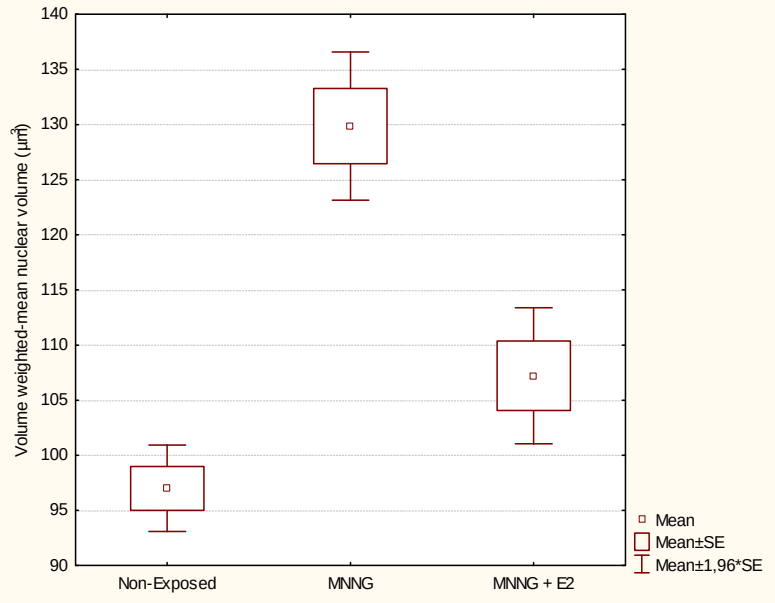

Figure 14 – Brown trout. Graphic plotting of the nuclear $\overline{v}_v$ estimated in normal hepatocytes from negative controls (Non-Exposed), MNNG, and MNNG plus estradiol (MNNG + E2) groups. The differences between all the groups were statistically significant ($p < 0.01$).

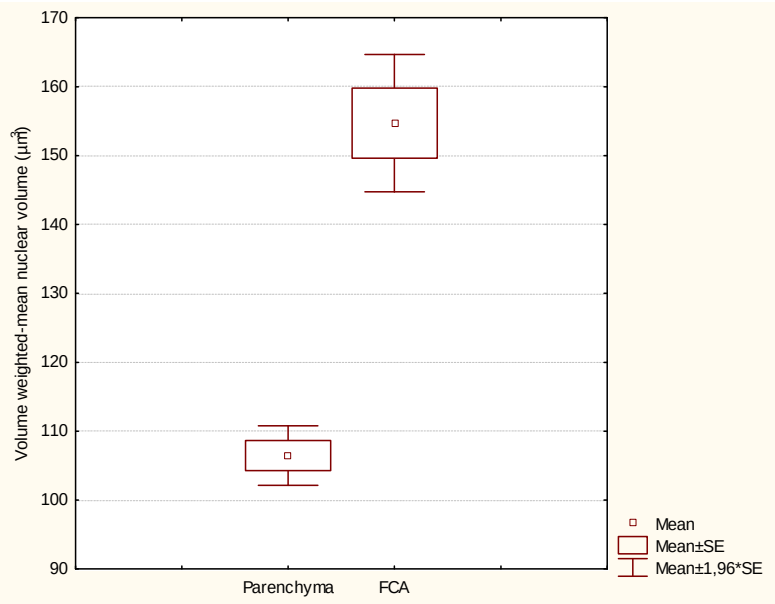

Figure 15 – Brown trout. Graphic plotting of the nuclear $\overline{v}_v$ estimated in the cells of the FCA and in hepatocytes of the internal control, *i.e.*, the normal surrounding tissue (Parenchyma). The nuclear $\overline{v}_v$ was significantly higher in FCA than in normal parenchyma ($p < 0.01$).

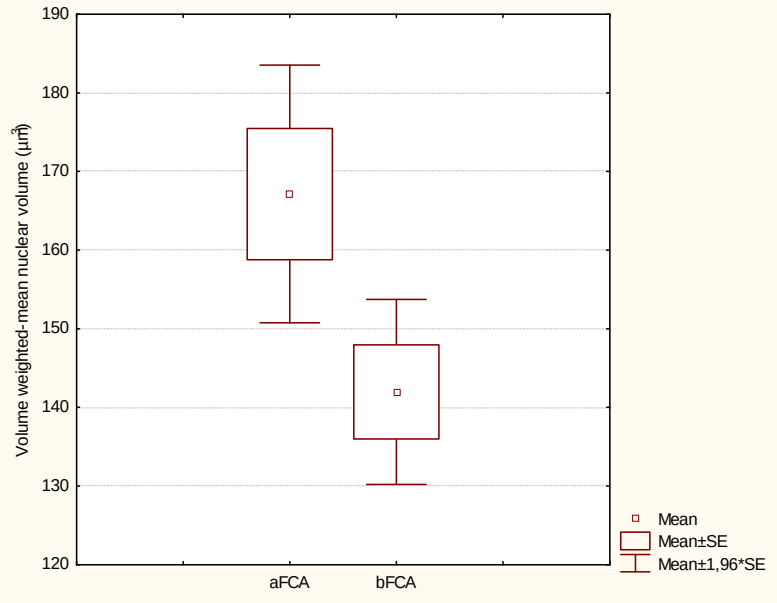

Figure 16 – Brown trout. Graphic plotting of the nuclear $\bar{v}_v$ estimated in the cells of the amphophilic (aFCA) and basophilic (bFCA) foci. The estimate in the aFCA was significantly higher (Student t-test for independent samples, $p < 0.05$).

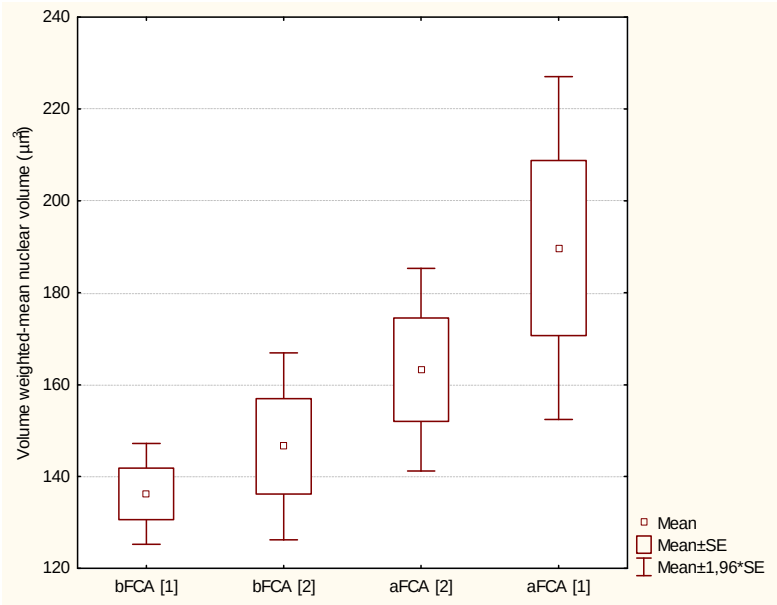

Figure 17 – Brown trout. Graphic plotting of the nuclear $\bar{v}_v$ estimated in the cells of the amphophilic (aFCA) and of the basophilic (bFCA) subtypes of foci. These were grouped in two ways: as the unique/exclusive subtype (aFCA[1], bFCA[1]); or as coexisting subtypes in the same liver (aFCA[2], bFCA[2]). A progression of this estimate existed from the exclusive bFCA[1] to the exclusive aFCA[1]. According to multiple comparisons testing, differences between the aFCA[1] and the bFCA[1] or bFCA[2] were significant ($p < 0.05$).

Part II – Dab

The classified 16 FCA, 8 neoplastic lesions and their respective normal parenchyma area pairs were submitted to stereological estimations of V_V (hepatocyte nucleus, parenchyma) and nuclear $\overline{v}_v$.

The V_V (hepatocyte nucleus, parenchyma) consistently increased from normal tissue to FCA and to neoplasia (Figures 18-20). The V_V obtained from the reported dubious case (a medium size lesion with some morphological atypia but without compression) was identified as a statistically significant outlier against the other estimated values of FCA (Grubbs´ test); consequently, this case was included in the group of neoplastic lesions. The one-way ANOVA revealed a statistically significant group effect ($p < 0.05$) (Figure 18) and, like for the V_V (hepatocyte nucleus, parenchyma), the nuclear $\overline{v}_v$ increased from normal parenchyma to FCA and to neoplasia (Figure 21, 22). However, the statistically significant differences obtained with the post-hoc Tukey's test were restricted to the following pairs: 1) normal parenchyma *versus* FCA ($p < 0.05$); 2) normal parenchyma *versus* neoplasia ($p < 0.05$).

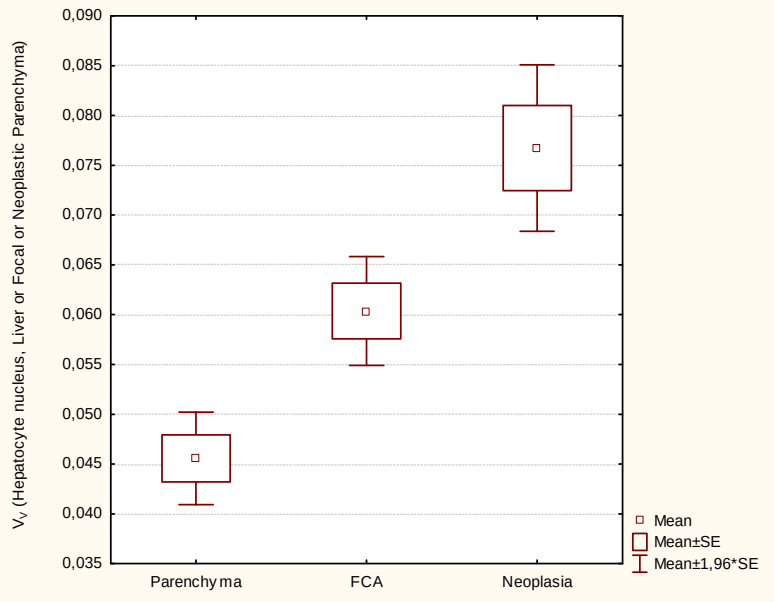

Figure 18 – Dab. Graphic plotting of the V_V of the hepatocyte nucleus estimated in normal parenchyma (Parenchyma), focal parenchyma (FCA) and neoplastic parenchyma (Neoplasia). After the one-way ANOVA, statistically significant differences ($p < 0.05$) between all the pairs were obtained under the Tukey's test.

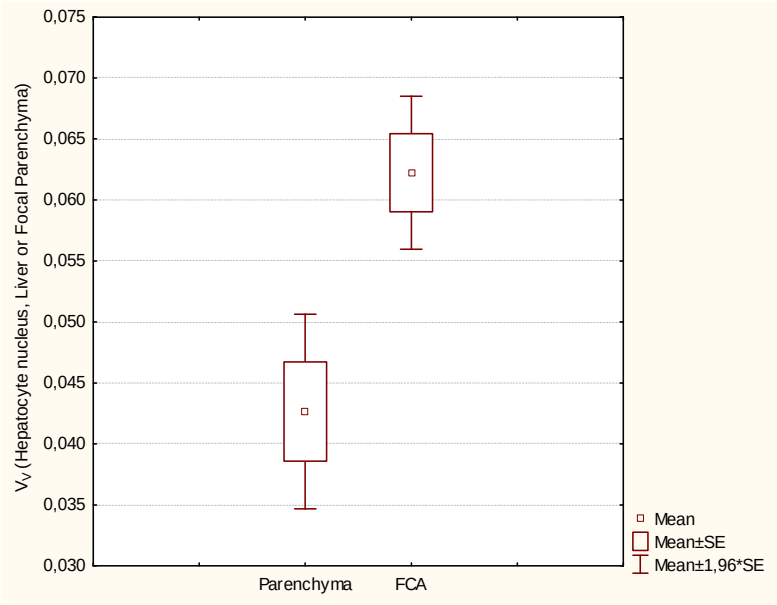

Figure 19 – Dab. Graphic plotting of the V_V (hepatocyte nucleus, parenchyma) in normal parenchyma (Parenchyma) and in FCA. The V_V was significantly higher in the FCA (Student's t-test for matched pair samples, $p < 0.01$).

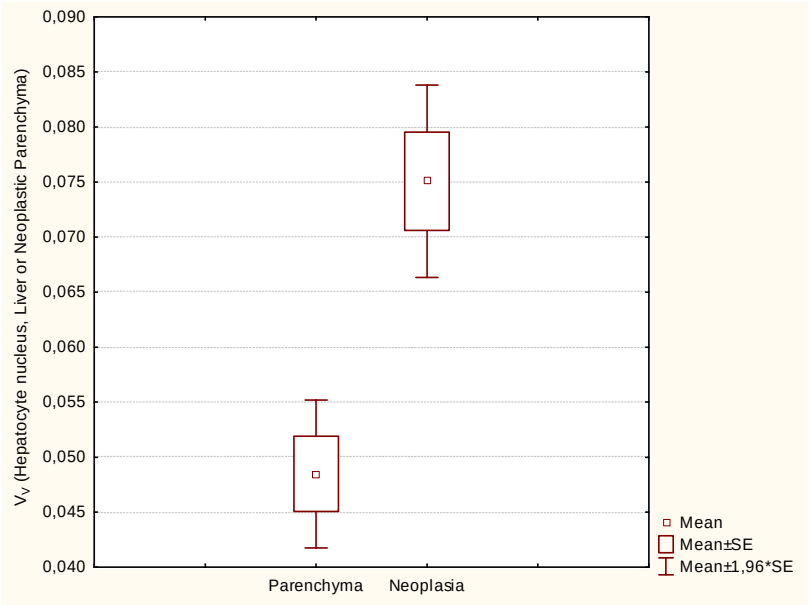

Figure 20 – Dab. Graphic plotting of the V_V (hepatocyte nucleus, parenchyma) in normal liver parenchyma (Parenchyma) and in neoplasia. The V_V was significantly higher in the neoplastic tissue (Student's t-test for matched pair samples, $p < 0.01$).

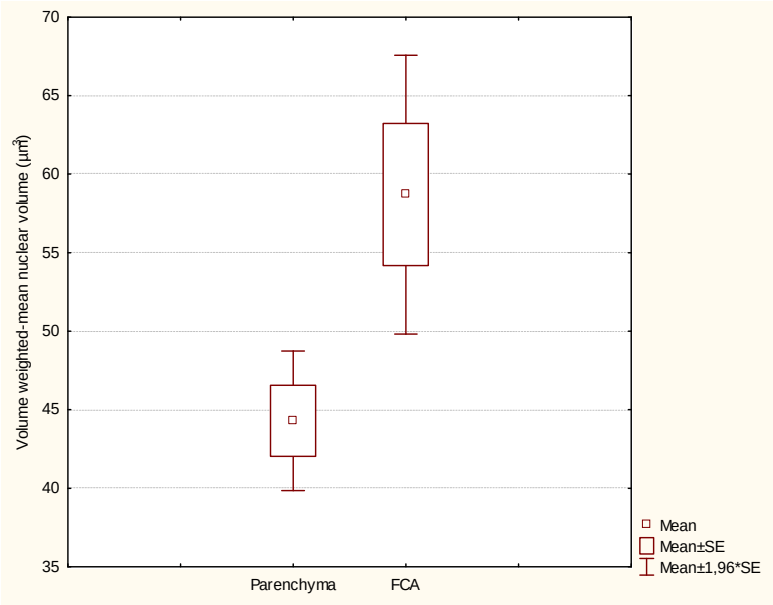

Figure 21 – Dab. Graphic plotting of the nuclear $\overline{v}_v$ in normal hepatocytes (Parenchyma) and in FCA. This stereological estimate was significantly higher in the FCA (Student's t-test for matched pair samples, $p < 0.05$).

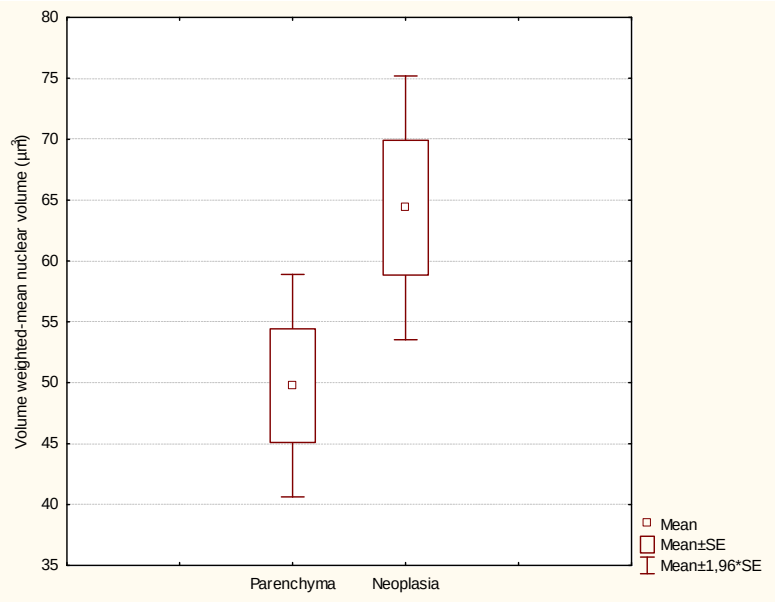

Figure 22 – Dab. Graphic plotting of the nuclear $\overline{v}_v$ in normal hepatocytes (Parenchyma) and in neoplasia. The mean value was significantly higher in the latter (Student's t-test for matched pair samples, $p < 0.05$).

DISCUSSION

General considerations

The teleost liver is one of the most sensitive organs for demonstrating morpho-physiological changes after exposure to environmental pollutants, and hepatocellular nodular lesions have been considered relevant histopathological biomarkers (Hinton and Couch 1998; Stentiford *et al*. 2003). In consequence, they can be used as proxies of both the animal and environmental health, and may be integrated in risk evaluation.

The classification of hepatocellular proliferative lesions varies between pathologists and the lack of consistency in the morphological criteria has been recognized for long (Broxup *et al*. 1988; Blazer *et al*. 2006). In this study we aimed to explore the utility of two stereological estimates for the differentiation of hepatocellular nodular lesions. For this purpose, we used two fish species, which can be regarded as a laboratory model (brown trout) and an environmental sentinel (dab) (Feist *et al*. 2004; Stentiford *et al*. 2005; Rocha *et al*. 2006). Our approach followed two sequential steps: 1) morphological classification of all the existing hepatocellular lesions in the samples, using a list of selected qualitative criteria; 2) quantitative measurements using software-assisted stereology, seeking for parameters and "cut-off values" with potential to assist in the differentiation (and logically also grading) of those lesions.

Qualitative histopathology

Regarding the observed lesions, the selected criteria allowed a definitive classification of all the cases, except for two (one case in trout and other case in dab); which does not mean other observers would reach the same diagnosis in every case. Comparing the samples of the two species, in those of the dab we observed a larger spectrum of lesions that included already established neoplasia — some with atypical architectural and cellular features that allowed the definition of malignancy. It is opportune to mention that the diagnosis of hepatocellular neoplasia in sentinel fish, including the dab of the North Sea, has been frequently reported (Vogelbein *et al*. 1990; Myers *et al*. 1991; Mikaelian *et al*. 1998; Koehler 2004; Stentiford *et al*. 2005). In contrast, all the lesions in the trout presented a normal arrangement of hepatocytes, with occasionally pronounced tubular formations — related with the enlargement of the sinusoids — a feature that has already been described in rodents (Koen *et al*. 1983). In our case we failed to observe nodular lesions that fulfilled the criteria of hepatocellular neoplasia in the initiator plus promoter (MNNG plus estradiol) exposed brown trout. This may be accounted by: 1) the adopted restrictive criteria, since all the major criteria had to be present in order to define neoplasia; 2) low frequency of neoplastic lesions resulting from this carcinogenesis

protocol; 3) not enough temporal lag between the exposure and the euthanasia. Regarding the frequency of neoplasia when using MNNG as initiator, Orner *et al*. (1996) reported that this compound administered to rainbow trout fries by water bath was a poor initiator of liver neoplasms; only when associated to large dose of a sex steroid promoter it produced a high incidence of liver tumors. Anyway, Orner *et al*. (1996) used a lower MNNG concentration (35 ppm) and time (7 months after initiation) that in our case. An increased temporal lag (between exposure and end of the experiment) allows more time to tumor development and it has been reported that the frequency of foci and neoplasia increases as a function of this time period (Cooke and Hinton 1999). In fact, malignant hepatocellular neoplasia has been diagnosed in laboratory fishes. For instance, using diethylnitrosamine (DEN) as an initiator, hepatocellular carcinoma was observed in medaka exposed: 1) in the larval period to 250 ppm of DEN and euthanatized 3 months later (Teh and Hinton 1998); 2) in juvenile age to higher doses of DEN for 4 weeks and sacrificed 9 months later (Boorman *et al*. 1997); 3) in adults exposed to a low dose (50 ppm) of DEN but for longer time (5 weeks) (Boorman *et al*. 1997); and in 189 day-old medaka sacrificed at 6 months post exposure to 100 mg/L of DEN (Brennan *et al*. 2001). In the case of the rainbow trout, malignant lesions were observed in animals exposed to diverse carcinogens in the embryo phase and euthanized 1 year later (Hendricks *et al*. 1984). It may be also questioned if different fish species differ in their sensitivity, namely considering that genetic and hereditary factors influence the individual resistance to carcinogenesis induced by N-nitrosamino compounds (Melhem *et al*. 1993).

Besides contaminant exposure, the occurrence of preneoplastic and neoplastic lesions in the wild has been related to the size, age and sex of fish, as well as exogenous factors, such as salinity (Au 2004). We did not have access to all that information for making an in depth analysis of the background situation for dab, but our primary goals were related with the hepatocellular lesions *per se* and (particularly) their quantitative morphological characteristics, disregarding the prevalence of each type of lesions and also their implications for the animal or inferences to the aquatic ecosystems health.

Regarding the classification of the lesions, we opted to consider the amphophilic as an independent subtype of the FCA in dab and in brown trout, following the criterion of Cooke and Hinton (1999) in medaka fish, even though it remains controversial for long (Boorman *et al*. 1997). In fact, this subtype has been well documented in human and rodent liver pathology (Su *et al*. 1997; Mayer *et al*. 2003). It has been even studied ultrastructurally in rodent models of hepatocarcinogenesis, being characterized by a strikingly high content of cristae-rich mitochondria wrapped in cisternae of the rough endoplasmic reticulum with a slightly increased number of peroxisomes (Mayer *et al*. 2003). The amphophilic FCA has

been induced (in experiments) predominantly by peroxisome proliferators, as dehydroepiandrosterone (Mayer *et al*. 2003) and their constituent cells demonstrated alterations in mitochondrial enzymes (Sukata *et al*. 2004). In contrast to this, some authors claimed that the amphophilic FCA designation should be avoided in fish and that lesions should be included in the basophilic subtype because their distinction is often unclear (Boorman *et al*. 1997). Anyway, in the studied fish species, the amphophilic subtype has been described — despite being rare in dab (Stentiford *et al*. 2003; Feist *et al*. 2004). Considering all, in this study we included the amphophilic subtype in the classification system because: 1) within the FCAs there were cells with increased acidophilia and randomly scattered cytoplasmic basophilia — this occurred in both species, but especially in the brown trout; 2) uniform criteria had to be used for the two species; and 3) it could provide a unique chance to look for eventual differential quantitative characteristics that could substantiate the morphology.

The classification of FCA usually includes the basophilic, eosinophilic, vacuolated and the clear cell subtype (Boorman *et al*. 1997). The vacuolated consists of hepatocytes with several medium to large lipid vacuoles within the cytoplasm and a marginal nucleus (Vogelbein *et al*. 1990; Boorman *et al*. 1997; Feist *et al*. 2004). In the clear cell FCA the ground glass appearance of the constitutive cells is the main feature (*i.e.,* slightly eosinophilic cytoplasm with glycogen and a centrally placed nucleus). We observed hepatocytes with different degrees of cytoplasmic vacuolization in the dab and brown trout livers, but failed to detect focal nodular lesions composed by cells with morphological features that satisfied the criteria of either vacuolated or clear cell FCA (Boorman *et al*. 1997; Feist *et al*. 2004). Regarding the dab, this result did not corroborate the reported high prevalence of the vacuolated subtype, but sustained that the clear cell subtype of FCA is rare in this species (Feist *et al*. 2004). Concerning the vacuolated FCA, we can hypothesize that the discrepancy between our findings and those of Feist *et al*. (2004) may be due to our relatively small number of samples (n=25) and/or to the difficulty in the detection of the vacuolated subtype in a liver composed of extremely vacuolated hepatocytes — a problem which has already been reported (Boorman *et al*. 199;, Feist *et al*. 2004). In a morphological perspective, the eosinophilic subtype was reported to have hypertrophied cells, whilst the basophilic FCA is said to be composed of hepatocytes that look smaller than normal (Vogelbein *et al*. 1990; Boorman *et al*. 1997; Bogovski *et al*. 1999; Blazer *et al*. 2006). In our qualitative analysis we did not detect differences in the size/volume of constitutive cells of FCA subtypes. This seems worth investigating with proper tools, as there is no clear explanation for these inconsistencies in the perceived hepatocyte size. Anyway, we wonder whether there are inter-specific differences also

about such issues or if various authors have diverse qualitative perceptions about the same reality.

It should be stressed that we observed a concomitant presence of different subtypes of FCAs in some brown trout and dab livers, as well as a simultaneous presence of FCA and neoplasia in a single dab liver. These have already been reported by Vogelbein *et al*. (1990) and Bogovski *et al*. (1999) and emphasize the progression of lesions from FCA to neoplasia. The presence of multiple morphological lesions in the same liver is an indirect evidence for the multistep progression of lesions in the carcinogenesis (Barrett 1993). In rodent models and marine fishes, cells with different degree of atypia have been observed in a same lesion — the so-called foci-in-foci (Bannasch 1986; Myers *et al.* 1991); this was not present in none of our lesions. In the progression to carcinogenesis, the basophilic subtype has been considered the main preneoplastic lesion in rodent models (Goldfarb *et al*. 1983; Bannasch 1986; Mayer *et al*. 2003) and in fish species (Hendricks *et al* 1984; Brown-Peterson *et al*. 1999; Blazer *et al*. 2006). However, in the single dab liver that present both FCA and neoplastic lesions, only an amphophilic FCA and adenoma were present; anyway, based on the material we have from dab we cannot discard the occurrence of the basophilic subtype in that liver.

In the livers of exposed brown trout we encountered a low frequency of eFCA, whereas in dab this subtype was as frequent as the bFCA and aFCA. The first observation is in accordance with preliminary literature on that species (Rocha *et al*. 2006), but contrasts with one of the first reports of experimentally induced liver lesions in the rainbow trout (Hendricks *et al*. 1984). Regarding the frequency seen in dab, it is not in agreement with Feist *et al*. (2004), who described eFCA as an occasional diagnostic lesion; this may also be explained by the low number of samples studied herein.

In our histopathological evaluation we did not observe signs of leukocyte infiltration (*i.e.* a presumable cellular immune response) in any eFCA or hepatocellular adenoma, nor the presence of giant multinucleated cells in the hepatocellular carcinomas — therefore, we failed to confirm previous reports in fish (Hendricks *et al*. 1984; Vogelbein *et al*. 1990; Feist *et al*. 2004; Blazer *et al*. 2006). We wonder whether the underlying reasons are connected to interspecific differences, wild *vs*. experimental scenarios, staging of the lesion(s), or other modelling factors. Another interesting finding was the presence of compression of surrounding parenchyma in all the neoplastic lesions of the dab, supporting that this criterion is important for the diagnosis of hepatocellular neoplasia (Boorman *et al*, 1997; Blazer *et al*. 2006; Feist *et al*. 2004). Only once there was a non-compressed nodule with architectural and cellular atypia. Feist *et al*. (2004) considered that lack of compression with architectural abnormalities can still drive to a diagnosis of

neoplasia, namely of an adenoma. It is noteworthy that in our case the non-compressive lesion rendered stereological measurements similar to those of neoplastic lesions, which enabled us to diagnose it as a hepatocellular adenoma. Even though it was a single case, it elucidates the potential utility of stereology in solving cases with morphologically dubious features. Further studies with a large number of borderline lesions are justified and needed in order to fully confirm this issue.

Stereological evaluation

Regarding the study design, we should stress that different types of controls were sampled (internal and external), in order to cope with the reported high intraspecies variability in fish (Rocha *et al*. 2009). Regarding the former, the use of the surrounding morphologically normal parenchyma for the comparative stereological estimations has been previously reported (Jack *et al*. 1990). These internal controls have several advantages: 1) they are easily accessible while doing the stereological measurements; 2) comparisons are not affected by inter-animal differences; 3) they are a comparable pair affected by a similar amount of shrinkage. This is relevant, because shrinkage markedly affects stereological measurements of volume (Sørensen 1992; Howard and Reed 1998). The potential disadvantage is that the normal parenchyma may be not as normal as expected, being eventually influenced by the ongoing liver pathology. As to the external control, it was only used for the brown trout because these were laboratory animals, exposed to a defined hepatocarcinogenesis protocol. In those animals, it was relevant to quantify (by stereology) the effect of the initiator (MNNG) alone and events associated with the promoter (estradiol). Additionally, it was important to establish if the normal appearing surrounding parenchyma (of exposed fish) had comparable stereological parameters to the normal parenchyma of non-exposed animals (negative control group); to our knowledge, this was never accessed before. In the brown trout we saw that differences existed in both the V_V (hepatocyte nucleus, parenchyma) and nuclear $\overline{v}_v$ between those normal appearing hepatocytes (*i.e.*, evaluated in internal *vs.* external controls). In this vein, the exclusive use of the ordinary parenchyma as an internal-control may represent a drawback, as the apparently normal hepatocytes seem indeed influenced (in still undetermined ways) by the presence of FCA in liver. Therefore, at this point we do recommend including external controls — non-exposed animals — in laboratory studies. In field studies the use of animals captured from areas without previously reported exposure to environmental xenobiotics might be an option.

Concerning the stereological parameters, it is noteworthy to recall that estimates of V_V (hepatocyte nucleus, parenchyma) are fractions, giving information about the volume of

nuclei in proportion of reference space. Increases in this parameter can always be due to a factual increase of the nuclear volume of each cell and/or to a higher number of cells (within the reference volume) (Howard and Reed 1998; Mandarim-de-Lacerda 2003). Alternatively, an increased V_V can result from a reduction of the reference space, for example due (in our context) to an eventual decrease in the volume of the cytoplasm without a corresponding decrease in the nuclear size; this is the so-called “reference trap” (Howard and Reed 1998). On the other hand, estimates of nuclear $\overline{v}_v$ combine information about 3D size with size variability and, consequently, directly and positively increase as a function of a greater diversity in nuclear volume — *i.e.*, nuclear pleomorphism or anisokaryosis (Sørensen 1992). In this study, it is important to point out that both the V_V and nuclear $\overline{v}_v$ were significantly different amongst hepatocellular lesions (disregarding the type) and normal surrounding parenchyma. Besides this, the two types of estimates in the normal parenchyma also differed among brown trout groups exposed to the initiator, initiator plus promoter and also in non-exposed fish. According to our findings, the exposure to initiator (MNNG) in the late phase of the embryonic life, associated or not with the exposure to a promoter (estradiol) during juvenile growth, leads to alterations in the hepatocyte nuclear morphology that can also be assess with the used stereological tools. These are major fundamental findings of our study, which have never been reported — to the best of our knowledge. Although we only studied two fish species, it is reasonable to assume that the differences in the V_V and the nuclear $\overline{v}_v$ will stand in other scenarios (*e.g.*, other fish species, exposure to other chemical compounds). In this vein, we can anticipate that these stereological estimations, besides providing statistically manageable data, seem clearly of use as a supportive method for the otherwise subjective histological diagnosis of hepatocellular nodular lesions in fish, like it has been recommended in different neoplastic lesions in humans (*e.g.,* Steiner *et al*. 1994; Fujikawa *et al*. 1995; Fujikawa *et al*. 2000).

It is believed that the estradiol and other estrogenic female hormones promote liver growth by increasing mitogenesis of hepatocytes, thus playing an important role in the sex-dependent hepatic morphology and in the development of tumoral lesions (Teh and Hinton 1998; Rocha *et al*. 2001; Rocha *et al*. 2009). Our results indicated that hepatocytes (outside lesions) have smaller V_V (hepatocyte nucleus, parenchyma) and greater nuclear $\overline{v}_v$ after the exposure to the initiator — this probably reflects a high nuclear size variability (Figure 23). The subsequent chronic exposure to the promoter decreased the nuclear $\overline{v}_v$ and marginally increased the cited V_V (a difference that was not statistically significant). It could be hypothesized that more numerous but smaller hepatocytes were present in those

livers (Figure 25). This hypothesis is supported by the theory that (at least under breeding cycles of seasonal breeders, like trout) estradiol promotes mitotic activity in liver, increasing the number of hepatocytes per unit volume of parenchyma, with a parallel decrease in the total volume of each hepatocyte (*i.e.,* the hepatocytes resulting from an accelerated mitotic activity are smaller than before) (Rocha *et al*. 2009). Moreover, our results are also in accordance with Grasl-Kraupp *et al*. (2000), who concluded that the hepatocyte turnover (integrating cell replication and apoptosis) is accelerated from the early onset of the hepatocarcinogenesis process.

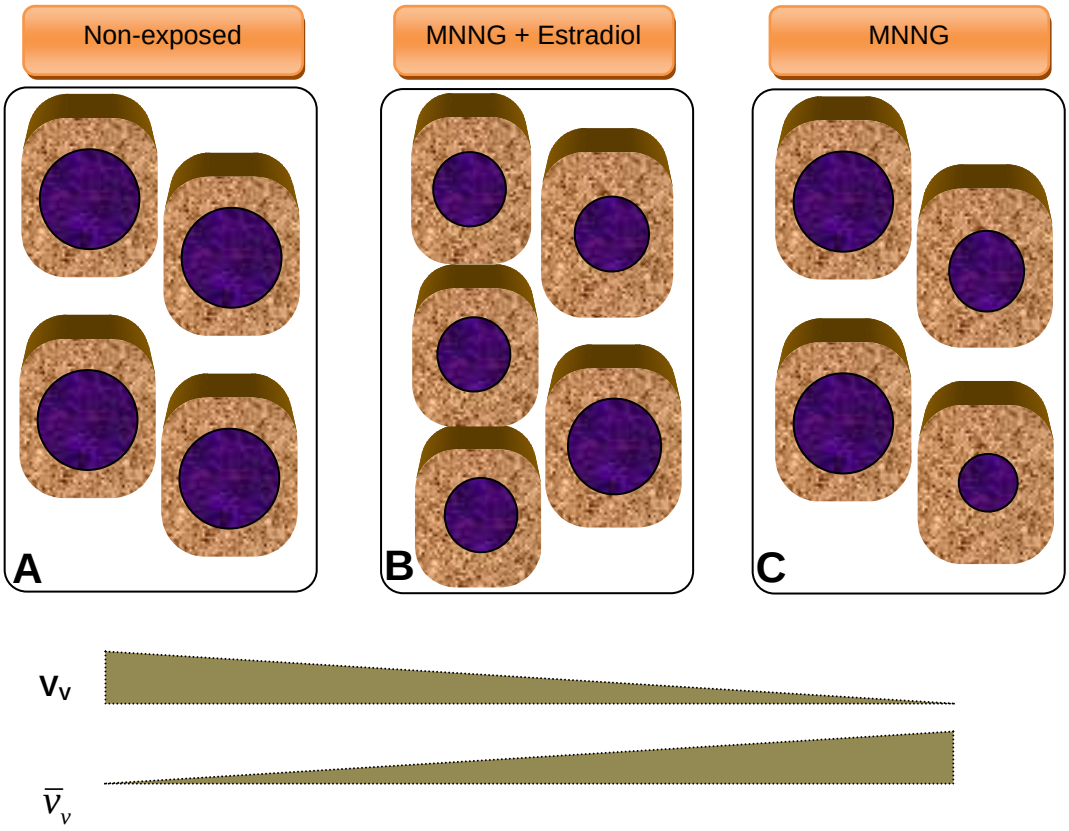

Figure 23 – Schematic representation of one explanatory hypothesis about the events that happened in the normal hepatocytes in non-exposed (A), in initiator plus promoter (MNNG + estradiol) (B) and initiator only (MNNG) (C) exposed brown trout; variations in V_V (hepatocyte nucleus, parenchyma) and $\overline{v}_v$ (hepatocyte nucleus) are depicted. In (A) the hepatocytes presented the highest V_V and smallest nuclear $\overline{v}_v$, the latter likely traducing low nuclear pleomorphism; in (B) the mitogenic outcome produced more but smaller hepatocytes that coexisted with hepatocytes similar to those present in (A); in (C), cells with variable nuclear volume and higher cytoplasmic:nuclear ratio appeared.

In MNNG plus estradiol exposed brown trout, the number of bFCA and aFCA allowed a statistical comparison: while the V_V (hepatocyte nucleus, parenchyma) did not differ, the nuclear $\overline{v}_v$ was significantly higher in aFCA, thus suggesting an increase in nuclear pleomorphism. When we split the bFCA and aFCA that appeared as the exclusive subtype from those coexisting in the same liver, this was further evident, since we found a progressive increase in the nuclear $\overline{v}_v$ from the exclusive bFCA to the exclusive aFCA (recall Figure 17). Facing that the highest values of the nuclear $\overline{v}_v$ were found in neoplastic lesions, this trend of an increasing $\overline{v}_v$ from the basophilic to the amphophilic subtype is surprising (because suggestive that bFCA preceded aFCA, which would precede neoplasia) and contrasts with the almost consensual hypothesis of the biological fate of bFCA. In fact, most authors suspect that the amphophilic phenotype is a precursor of the basophilic phenotype. It has been supposed that the bFCA represent the last stage before the transformation into neoplastic lesions (*e.g,* Goldfarb *et al*. 1983; Hendricks *et al*. 1984; Brown-Peterson *et al*. 1999; Mayer *et al*. 2003). Based in our results, the increased $\overline{v}_v$ in aFCA, either due to larger nuclei or to greater nuclear pleomorphism, favours the view of this lesion as truly preneoplastic.

As already mentioned, in dab samples we faced a larger spectrum of lesions, including neoplasia, which enabled us to perform a statistical comparison between the normal, focal and neoplastic hepatocytes. The two stereological estimates increased in a linear manner from normal parenchyma to FCA and to neoplasia; however only the variation of V_V (hepatocyte nucleus, parenchyma) was statistically significant. The estimates of nuclear $\overline{v}_v$ could satisfactorily separate the normal parenchyma from FCA and from neoplasia, but the subtypes of foci and neoplasia could not be differentiated (within the limits of this study). Due to the small number of neoplastic lesions studied (8 cases), definitive conclusions cannot be withdrawn at this point and more samples would have to be studied in order to fully establish the nuclear $\overline{v}_v$ estimate as a discriminative tool to differentiate hepatocellular proliferative lesions.

CONCLUSIONS

In the present study we assessed qualitatively and quantitatively various hepatocellular nodular lesions in two different fish species with distinct background contexts. The stereological tools used supported the criteria selected for our classification system. Except for eFCA, we were able to stereologically discriminate the categories of lesions, with statistically significant differences. According to our findings, the foundations of the diagnosis of hepatocellular neoplasia should include the simultaneous observation of compression of the surrounding parenchyma, increased cellularity and, at last, nuclear pleomorphism. If, in one hand, the use of compression alone as a definitive criterion for neoplasia is the main drawback of some of the existing classification systems, in the other hand, the absence of this compression should not exclude a diagnosis of neoplasia. We demonstrated that aFCA and bFCA can be differentiated not only by their staining differences, but also by the nuclear $\bar{v}_v$. This latter further supported that the aFCA should be regarded as a separate entity in the classification of hepatocellular lesions, namely in the brown trout (and very unlikely only in this species). Overall, both the V_V (hepatocyte nucleus, parenchyma) and nuclear $\bar{v}_v$ seem useful for discriminating normal parenchyma, from foci and neoplastic lesions, either in experimental settings or in biomonitoring. Although a larger number of cases need to be studied, the results are promising — especially regarding their use in cases with doubtful histological features. Moreover, the discriminative power revealed by the stereological parameters sustains there is potential of their use to grade the lesions and disclose dose-effects relations.

Besides the utility of the used stereological tools in studying the hepatocellular lesions, we also reported quantitative nuclear differences in normal appearing hepatocytes in all steps of induced hepatocarcinogenesis in a fish model — this is another novel finding of this study, which warns that the healthy liver tissue may structurally and, even functionally, differ when comparing fish with and without ongoing hepatocarcinogenesis.

In summary, we attained all the main objectives of this study by: 1) classifying all the proliferative hepatocellular lesions, using a combination of stringent histopathological diagnostic criteria; 2) estimating the volume density of the liver cell nucleus and nuclear $\bar{v}_v$ in each classified lesion; 3) disclosing differences in the stereological estimations obtained from the normal uninvolved liver parenchyma, FCA and neoplastic lesions (whenever these existed); 4) revealing differences between subtypes of FCA, and highlighting the cases where the estimates paralleled the morphological distinction; 5) demonstrating that the volume density of the nucleus and the nuclear $\bar{v}_v$ are useful for the classification scheme of the lesions, showing potential for establishment of cut-offs.

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REFERENCES

Argyle DJ, Khanna C (2007). Tumor biology and metastasis. In: Withrow SJ, Vail DM (eds.). Withrow & MacEwen Small Animal Clinical Oncology, 4th Edition, Saunders Elsevier, St. Louis, Missouri, USA, p 31-53.

Au DMT (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Mar Pollut Bull 48, 817-834.

Bailey SG, Williams DE, Hendricks JD (1996). Fish models for environmental carcinogenesis: the rainbow trout. Environ Health Perspect 104, 5-21.

Bannasch P (1986). Pre-neoplastic lesions as end points in carcinogenicity testing. I – hepatic preneoplasia. Carcinogenesis 7, 689-695.

Barrett JC (1993). Mechanisms of multistep carcinogenesis and carcinogen risk assessment. Environ Health Perspect 100, 9-20.

Beckman RA, Loeb LA (2006). Efficiency of carcinogenesis with and without a mutator mutation. Proc Natl Acad Sci 103, 14140-14145.

Binder M, Dolezal I, Wolff K, Pehamberger H (1992). Stereologic estimation of volume-weighted mean nuclear volume as a predictor of prognosis in "thin" malignant melanoma. J Invest Dermatol 99, 180-183.

Blazer VS, Fournie JW, Wolf JC, Wolfe MJ (2006). Diagnostic criteria for proliferative hepatic lesions in brown bullhead *Ameiurus nebulosus*. Dis Aquat Organ 72, 19-30.

Blum HE, Spangenberg HC (2007). Hepatocellular carcinoma: an update. Arch Iran Med 10, 361-71.

Boorman GA, Botts S, Bunton TE, Fournie JW, Harshbarger JC, Hawkins WE, Hinton DE, Jokinen MP, Okihiro MS, Wolfe MJ (1997). Diagnostic criteria for degenerative, inflammatory, proliferative nonneoplastic and neoplastic liver lesions in medaka (*Oryzias latipes*): consensus of a National Toxicology Program Pathology Working Group. Toxicol Pathol 25, 202-210.

Brennan LM, Boncavage-Hennessey EM, Wolfe MJ, Toussaint MW, Dennis WE, Rosencrance AB, Gardner HS Jr (2001). .An in vivo method for using 5-bromo-2'-deoxyuridine (BrdU) as a marker of chemically-induced hepatocellular proliferation in the Japanese medaka (*Oryzias latipes*). Toxicol Pathol 29, 387-397.

Brown-Peterson NJ, Krol RM, Zhu Y, Hawkins WE (1999). N-nitrosodiethylamine initiation of carcinogenesis in Japanese medaka (*Oryzias latipes*): hepatocellular proliferation,

toxicity, and neoplastic lesions resulting from short term, low level exposure. Toxicol Sci 50, 186-194.

Broxup BR, Valli VE, Losos GL, Percy DH, Farber E, McMillan I (1988). Morphometric evaluation of hepatocellular proliferative lesions in the rat liver. Toxicol Pathol 16, 401-417.

Bunton TE (1996). Experimental chemical carcinogenesis in fish. Toxicol Pathol 24, 603-618.

Campbell HA, Pitot HC, Potter VR, Laishes BA (1982). Application of quantitative stereology to the evaluation of enzyme-altered foci in rat liver. Cancer Res 42, 465-472.

Cooke JB, Hinton DE (1999). Promotion by 17b-estradiol and b-hexachlorocyclohexane of hepatocellular tumors in medaka, *Oryzias latipes.* Aquat Toxicol 45, 127-145.

Farazi PA, DePinho RA (2006). Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 6, 674-687.

Fearon ER (1999). Cancer progression. Curr Biol 2, R873-5

Feist SW, Lang T, Stentiford GD, Köhler A (2004). Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limanda limanda L.)* and flounder (*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences, Nº 38, p 38-42.

Fujikawa K, Matsui Y, Kobayashi T, Miura K, Oka H, Fukuzawa S, Takeuchi H (2000). Predicting pathological stage of localized prostate cancer using volume weighted mean nuclear volume. J Urol 164, 1587-1590.

Fujikawa K, Sasaki M, Aoyama T, Itoh T, Yoshida O (1995). Prognostic criteria in patients with prostate cancer: correlation with volume weighted mean nuclear volume. J Urol 154, 2123-2137.

Gibbons CH, Illigens BM, Wang N, Freeman R (2009). Quantification of sweat gland innervation: a clinical-pathologic correlation. Neurology 28, 1479-86.

Giordano A, de Falco G, Rubin E, Rubin R (2008). Neoplasia. In: Rubin E and Reisner HM (eds.). Essential of Rubin's Pathology, 5th Edition, Lippincott Williams & Wilkins, Philadelphia, USA, p 230-277.

Globocan 2008. Cancer incidence and mortality worldwide in 2008. http://globocan.iarc.fr/ (accessed in 25 of June of 2010).

Goldfarb S, Pugh TD, Koen H, He YZ (1983). Pre-neoplastic and neoplastic progression during hepatocarcinogenesis in mice injected with diethylnitrosamine in infancy. Environ Health Perspect 50, 149-161.

Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD (2008). Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. World J Gastroenterol 21, 4300-4308.

Grasl-Kraupp B, Luebeck G, Wagner A, Löw-Baselli A, de Gunst M, Waldhör T, Moolgavkar S, Schulte-Hermann R (2000). Quantitative analysis of tumor initiation in rat liver: role of cell replication and cell death (apoptosis). Carcinogenesis 21, 1411-1421.

Gundersen HJ, Jensen EB (1985). Stereological estimation of the volume-weighted mean volume of arbitrary particles observed on random sections. J Microsc 138 (Pt 2), 127-42.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. Cell 100, 57-70.

Hamilton SR, Aaltonen LA (2000). Tumours of the liver and intrahepatic bile ducts. In: Hamilton SR, Aaltonen LA (Eds.): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Digestive System. IARC Press, Lyon, France, p 157-202.

Hardman RC, Volz DC, Kullman SW, Hinton DE (2007). An in vivo look at vertebrate liver architecture: three-dimensional reconstructions from medaka (*Oryzias latipes*). Anat Rec (Hoboken) 290, 770-782.

Hendricks JD, Meyers TR, Shelton DW (1984). Histological progression of hepatic neoplasia in rainbow trout (*Salmo gairdneri*). Natl Cancer Inst Monogr 65: 321-336.

Hinton D, Couch J 1998. Architectural pattern, tissue and cellular morphology in livers of fishes: relationship to experimentally-induced neoplastic responses. EXS 86, 141-164.

Howard CV, Reed MG (1998). Estimation of component volume and volume fraction. In: Howard CV, Reed MG (eds.). Unbiased Stereology, Bios Scientific Publishers, Oxford, United Kingdom, p. 55-68.

Jack EM, Bentley P, Bieri F, Muakkassah-Kelly SF, Stäubli W, Suter J, Waechter F, Cruz-Orive LM (1990). Increase in hepatocyte and nuclear volume and decrease in the population of binucleated cells in preneoplastic foci of rat liver: a stereological study using the nucleator method. Hepatology 11, 286-297.

Jack EM, Cruz-Orive LM, Waechter F, Stäubli W (1989). Unbiased estimation of cell and nuclear volume in preneoplastic and uninvolved tissue from the same rat liver using the nucleator. Acta Sterol 8, 257-262.

Koehler A (2004). The gender-specific risk to liver toxicity and cancer of flounder (*Platichthys flesus* (L.)) at the German Wadden Sea coast. Aquat Toxicol 70, 257-276.

Koen H, Pugh TD, Goldfarb S (1983). Hepatocarcinogenesis in the mouse. Combined morphologic-stereologic studies. Am J Pathol 112, 89-100.

Kumar V, Cotran RS, Robbins SL (2003). Neoplasia, In: Kumar V, Cotran RS, Robbins Sl (eds.). Robbins Basic Pathology, 7th Edition, Saunders, Philadelphia, USA. p 260-327.

Ladekarl M (1998). Objective malignancy grading: a review emphasizing unbiased stereology applied to breast tumors. APMIS Suppl 79, 1-34.

Law JM (2001). Mechanistic considerations in small fish carcinogenicity testing. ILAR J 42, 274-284.

Leong TY, Leong AS (2005). Epidemiology and carcinogenesis of hepatocellular carcinoma. HPB (Oxford) 7, 5-15.

Liu Z, Kullman SW, Bencic DC, Torten M, Hinton DE (2003). Ras oncogene mutations in diethylnitrosamine-induced hepatic tumors in medaka (*Oryzias latipes*), a teleost fish. Mutat Res 539, 43-53.

Loeb LA, Harris CC (2008). Advances in chemical carcinogenesis: a historical review and prospective. Cancer Res 68, 6863-6872.

Luch A (2005). Nature and nurture - lessons from chemical carcinogenesis. Nat Rev Cancer 5, 113-125.

Mandarim-de-Lacerda CA (2003). Stereological tools in biomedical research. An Acad Bras Cienc 75, 469-486.

Mayer D, Forstner K, Kopplow K (2003). Induction and modulation of hepatic preneoplasia and neoplasia in the rat by dehydroepiandrosterone. Toxicol Pathol 31, 103-112.

Melhem MF, Kunz HW, Gill TJ3rd (1993). A major histocompatibility complex-linked locus in the rat critically influences resistance to diethylnitrosamine carcinogenesis. Proc Natl Acad Sci 90, 1967-1971.

Mikaelian I, de Lafontaine Y, Menard C, Tellier P, Harshbarger J, Martineau D (1998). Neoplastic and nonneoplastic hepatic changes in Lake Whitefish (*Coregonus clupeaformis*) from the St. Lawrence River, Quebec, Canada. Environ Health Perspect 106, 179-183.

Minamoto T, Mai M, Ronai Z (1999). Environmental factors as regulators and effectors of multistep carcinogenesis. Carcinogenesis 20, 519-527.

Modiano JF, Breen M (2007). The etiology of cancer. In: Withrow SJ, Vail DM (eds.). Withrow & MacEwen Small Animal Clinical Oncology, 4th Edition, Saunders Elsevier, St. Louis, Missouri, USA, p 3-19.

Montalto G, Cervello M, Giannitrapani L, Dantona F, Terranova A, Castagnetta LA (2002). Epidemiology, risk factors, and natural history of hepatocellular carcinoma. Ann N Y Acad Sci 963, 13-20.

Myers MS, Landahl JT, Krahn MM, McCain BB (1991). Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the U.S. West Coast. Environ Health Perspect 90, 7-15.

Newell P, Villanueva A, Friedman SL, Koike K, Llovet JM (2008). Experimental models of hepatocellular carcinoma. J Hepatol 48, 858-879.

Ni YH, Chang MH, Huang LM, Chen HL, Hsu HY, Chiu TY, Tsai KS, Chen DS (2001). Hepatitis B virus infection in children and adolescents in a hyperendemic area: 15 years after mass hepatitis B vaccination. Ann Intern Med 6, 796-800.

Orner GA, Hendricks JD, Arbogast D, Williams DE (1996). Modulation of N-methyl-N'-nitro-nitrosoguanidine multiorgan carcinogenesis by dehydroepiandrosterone in rainbow trout. Toxicol Appl Pharmacol 141, 548-554.

Okihiro MS, Hinton DE (1999). Progression of hepatic neoplasia in medaka (*Oryzias latipes*) exposed to diethylnitrosamine. Carcinogenesis 20, 933-940.

Okuda H (2007). Hepatocellular carcinoma development in cirrhosis. Best Pract Res Clin Gastroenterol 21,161-173.

Oliveira PA, Colaço A, Chaves R, Guedes-Pinto H, De-La-Cruz PLF, Lopes C (2007). Chemical carcinogenesis. An Acad Bras Cienc 79, 593-616.

Philip PA, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C (2005). Phase II study of Erlotinib (OSI-774) in patients with advanced hepatocellular cancer. J Clin Oncol 23, 6657-6663.

Pitot HC, Goldsworthy TL, Moran S, Kennan W, Glauert HP, Maronpot RR, Campbell HA (1987). A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. Carcinogenesis 8, 1491-1499.

Rocha E, Marcos R, Galante MH, Monteiro RAF, Hinton DE (2006). Preneoplastic foci and benign neoplasms in brown trout – preliminary data on their induction by MNNG and promotion by estradiol and alkylphenols. Mar Environ Res 62, S59-60.

Rocha E, Monteiro RA, Oliveira MH, Silva MW (2001). The hepatocytes of the brown trout (*Salmo trutta f. fario*): a quantitative study using design-based stereology. Histol Histopathol 16, 423-437.

Rocha E, Monteiro RAF (1999). Histology and cytology of fish liver: a review. In: Saksena DN (ed.). Ichthyology: recent research advances. Science Publishers, Enfield, USA, p 321-344.

Rocha E, Rocha MJ, Galante MH, Silva MW, Monteiro RAF (2009). The hepatocytes of the brown trout (*Salmo trutta* f. *fario*): a stereological study of their number and size during the breeding cycle. Ichthyol Res 56, 43–54.

Sàfrány L, Tulassay Z, Walton E (1970). Pathological importance of changes in the nuclear volume of liver cells. Orv Hetil 111, 1449-1452.

Sarasin A (2003). An overview of the mechanisms of mutagenesis and carcinogenesis. Mutat Res 544, 99-106.

Schwarz M, Buchmann A, Bock KW (1995). Role of cell proliferation at early stages of hepatocarcinogenesis. Toxicol Lett 82-83, 27-32.

Smart RC (2004). Chemical carcinogenesis. In: Hodgson E (ed.). A textbook of modern toxicology, 3th Edition, John Wiley & Sons Inc, New York, USA, p 225-250.

Soda T, Fujikawa K, Ito T, Sasaki M, Nishio Y, Miyakawa M (1999). Volume-weighted mean nuclear volume as a prognostic factor in renal cell carcinoma. Lab Invest 79, 859-867.

Sørensen FB (1992). Stereological estimation of the mean and variance of nuclear volume from vertical sections. J Microsc 162 (Pt 2), 203-229.

Steiner A, Binder M, Mossbacher U, Wolff K, Pehamberger H (1994). Estimation of the volume-weighted mean nuclear volume discriminates Spitz's nevi from nodular malignant melanomas. Lab Invest 70, 381-385.

Stentiford GD, Viant MR, Ward DG, Johnson PJ, Martin A, Wenbin W, Cooper HJ, Lyons BP, Feist SW (2005). Liver tumors in wild flatfish: a histopathological, proteomic, and metabolomic study. OMICS 9, 281-299.

Stine CB, Smith DL, Vogelbein WK, Harshbarger JC, Gudla PR, Lipsky MM, Kane AS (2004). Morphometry of hepatic neoplasms and altered foci in the mummichog, *Fundulus heteroclitus*. Toxicol Pathol 32, 375-383.

Su Q, Bannasch P (2003). Relevance of hepatic preneoplasia for human hepatocarcinogenesis. Toxicol Pathol 31, 126-133.

Su Q, Benner A, Hofmann WJ, Otto G, Pichlmayr R, Bannasch P (1997). Human hepatic preneoplasia: phenotypes and proliferation kinetics of foci and nodules of altered hepatocytes and their relationship to liver cell dysplasia. Virchows Arch 431, 391-406.

Sukata T, Uwagawa S, Ozaki K, Sumida K, Kikuchi K, Kushida M, Saito K, Morimura K, Oeda K, Okuno Y, Mikami N, Fukushima S (2004). alpha(2)-Macroglobulin: a novel cytochemical marker characterizing preneoplastic and neoplastic rat liver lesions negative for hitherto established cytochemical markers. Am J Pathol 165, 1479-1488.

Tasca C, Enachescu D, Crăciun E (1970). Practical application of the karyo-interkaryometric method in pathology. Arkh Patol 32, 66-69.

Teh SJ, Hinton DE (1998). Gender-specific growth and hepatic neoplasia in medaka (*Oryzias latipes*). Aquat Toxicol 41, 141-159.

Vogelbein WK, Fournie JW, Van Veld PA, Huggett RJ (1990). Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. Cancer Res 50, 5978-5986.

Watson JD, Crick FH (1953). Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 25, 737-738.

Weibel ER, Hsia CCW, Ochs M (2007). How much is there really? Why stereology is essential in lung morphometry. J Appl Physiol 102, 459-467.

Xu Y, Pitot HC (2006). A software package to improve image quality and isolation of objects of interest for quantitative stereology studies of rat hepatocarcinogenesis. Comput Methods Programs Bimed 81, 236-245.

Yamagiwa K, Ichikawa KJ (1918). Experimental study of the pathogenesis of carcinoma. J Cancer Res 3, 1-29.

Yeo CJ (1999). Tumor suppressor genes: a short review. Surgery 125, 363-366.

Yörükoglu K, Aktas S, Güler C, Sade M, Kirkali Z (1998). Volume-weighted mean nuclear volume in renal cell carcinoma. Urology 52, 44-47.