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Xenoestrogenic and androgenic impacts on carcinogenesis onset – A mechanistically look at a controversial issue recurring to diethylnitrosamine induced carcinogenesis in zebrafish larvae

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Dissertation for the degree of Master in Environmental Contamination and Toxicology, submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto (ICBAS – UP)

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'Man is nothing but what education makes of him.' Immanuel Kant

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumour worldwide and accounts for about half a million deaths each year due to scarce treatment options. Numerous compounds, both carcinogenic such as diethylnitrosamine (DEN) and non-carcinogenic, such as hormones, are able to induce, promote or modulate this type of tumour, which occurs more frequently and more severely in males than females, both in humans and in model organisms. This is an issue that is not yet fully understood, since there is a disparity of results between studies regarding the modulatory role of androgens and oestrogens, and particularly whether they act as promoters or protectors.

Several studies have been focused on the impact of an HCC on lipid metabolism. This increased number of studies suggests that this linkage is gaining more attention by the researchers.

Thus, this study aims to contribute for clarifying if androgenic and estrogenic stimuli may model impacts of an HCC initiator, particularly at the early stages of initiation, and also to evaluate the effects of HCC on lipid metabolism, particularly the pathways that are altered.

To achieve this, zebrafish embryos (*Danio rerio*) were exposed for 48 hours to DEN, DEN with testosterone (TEST), DEN with ethinylestradiol (EE2) and to solvent only (SC). Histological techniques and biometric analysis on selected targets were performed to understand if there are morphological changes after the respective treatments. Quantitative Real-Time PCR (RT-qPCR) was used to evaluate the expression of a selection of genes from both lipid (*acaa2, elovl1a, lipca, scd*) and hepatocarcinogenesis pathways (*mapk1, myca, mycb, p21*).

In the morphological analysis, differences were detected between groups in several biometric parameters (especially in those containing the tail) and in hepatic volume. There is a clear slowdown in the development of exposed larvae and an increase in liver volume on the group of animals that were exposed to the carcinogen alone, suggesting a higher cell proliferation or cell hypertrophy. No differences in larvae weight were reported. Regarding molecular analysis, there was an increase in the mRNA levels of *acaa2* in DEN group and of gene *p21*, whose action depends on its cellular localization, in all the groups exposed to the carcinogenic agent.
The expression levels of the other tested genes were not significantly altered by any treatment.

In sum, this study concludes that there is, in fact, an impact of the carcinogen on the morphology of exposed larvae. However, molecular analysis did not allow us to conclude about the disparity that exists between the presence of androgens and the presence of oestrogens. Additionally, the increased mRNA levels of *acaa2* suggests that the β-oxidation pathway is increased due to the energy demand of transformed cells.

KEYWORDS Hepatocellular carcinoma; Zebrafish; Diethylnitrosamine; Testosterone; Ethinylestradiol

RESUMO

O carcinoma hepatocelular (CHC) é o quinto tumor maligno mais comum no mundo inteiro e é responsável por cerca de meio milhão de mortes todos os anos, devido às escassas opções de tratamento. Inúmeros compostos, quer agentes carcinogénicos, como a dietilnitrosamina (DEN), quer não carcinogénicos, como as hormonas, são capazes de induzir o aparecimento deste tumor, que ocorre mais frequentemente e de forma mais severa em indivíduos do sexo masculino (do que nos do sexo feminino), quer em humanos, quer em organismos modelo. Esta é uma questão que ainda não está completamente esclarecida, uma vez que existe disparidade de resultados entre estudos em relação ao papel dos estrogénios e dos androgénios, se promotor ou protetor.

Adicionalmente, tem aumentado, por parte da comunidade científica, o interesse em entender de que modo o aparecimento de CHC influencia o metabolismo lipídico.

Assim, este estudo tem como objetivos clarificar se estímulos androgénicos e estrogénicos podem modelar os impactos de um iniciador tumoral, e, ainda, perceber qual o efeito do aparecimento de um HCC no metabolismo lipídico, nomeadamente que vias são alteradas. Para isso, embriões de peixe-zebra (*Danio rerio)* foram expostos durante 48h a DEN, DEN com testosterona (TEST), DEN com etinilestradiol (EE2) e ao solvente (SC). Algumas técnicas histológicas e análises biométricas foram feitas com o objetivo de entender se há alterações morfológicas devido à exposição a um agente carcinogénico. PCR quantitativo em Tempo-Real (RT-qPCR) foi utilizado para avaliar a expressão dos genes selecionados, quer das vias lipídicas (*acaa2, elovl1a, lipca, scd*), quer das vias da hepatocarcinogénese (*mapk1, myca, mycb, p21*).

Na parte morfológica, foram detetadas diferenças em vários parâmetros biométricos (nomeadamente naqueles que incluem a cauda) e no volume hepático entre grupos. É registado um claro atraso no desenvolvimento dos peixes expostos e um aumento do volume hepático no grupo de peixes exposto ao agente carcinogénico sozinho, sugerindo uma maior proliferação celular ou hipertrofia celular. Nenhumas diferenças significativas no peso das larvas foram reportadas. Em relação à análise molecular, houve um aumento do nível de mRNA de *acaa2* no grupo DEN e do gene *p21*, cuja ação depende da sua localização celular, em todos

os grupos expostos. Os níveis de expressão dos outros genes testados não foram significativamente alterados por nenhum tratamento.
Em suma, este estudo concluiu que, de facto, a presença do agente carcinogénico influencia a morfologia dos peixes expostos. Contudo, a análise molecular não permitiu concluir sobre a disparidade que existe entre a presença de androgénios e a presença de estrogénios. O aumento dos níveis de mRNA de *acaa2* sugere que a via da β-oxidação está aumentada devido à energia que as células transformadas necessitam.

PALAVRAS-CHAVE Carcinoma hepatocelular; Zebrafish; Dietilnitrosamina; Testosterona; Etinilestradiol

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1. Introduction

1.1. Tumours and HCC

Normal cells can become transformed cells by the action of an initiator, a compound capable of inducing changes. If the DNA repair mechanisms and the apoptosis do not work properly, these transformed cells can proliferate and give origin to neoplasms. These neoplasms can be benign, or, by progression, they can evolve to a cancer – malignant neoplasm.

Cancer is a group of diseases well known by society, responsible for several deaths per day worldwide and is characterized by the uncontrolled division of non-normal (cancerous) cells. Mutations in this kind of cells can lead to the activation of oncogenes and inhibition of tumour suppressors, threatening the common balance between the genesis of new cells and cell death. If programmed cell-death (apoptosis) is compromised, along with other events, the malignant cells can travel through the body by the process of metastization (Santos *et al.*, 2017).

Hanahan and Weinberg (2000) reported that cancer cells share some features – called 'the six hallmarks of cancer' -, like: (1) sustained angiogenesis, (2) tissue invasion and metastasis, (3) limitless replicative potential, (4) evading apoptosis, (5) self-sufficiency in growth signals and (6) insensitivity to anti-growth signals.

Various types of cells can become cancer cells and have all the features referred above, and depending on the localization of these cells, there are different types of malignant tumours: carcinoma, formed from epithelial cells; sarcoma, started from connective tissue, such as bones, cartilage, and nerves; germ cell tumours, formed from cells that give life, sperm and egg cells; blastoma, originated from embryonic tissue (Cooper, 2000).

Hepatocellular carcinoma (HCC) affects the liver and it is the fifth more common malignant tumour worldwide and is responsible for 500.000 deaths per year, due to the limited efficacity of the treatment options (Parkin *et al.*, 2001; Kalra *et al.*, 2008; Barone *et al.*, 2009). It is one of the most studied cancers due to the crucial physiological role that the liver plays, since it is involved in several essential processes, like the efficient uptake of amino acids, carbohydrates, bile acids, cholesterol, proteins, lipids and vitamins for storage and subsequent metabolism, detoxification, blood volume regulation, and hormones secretion (Lemaigre & Zaret, 2004; Malarkey *et al.*, 2005; Zorn, 2008; Si-Tayeb *et al.*, 2010; Lu *et al.*, 2011). HCC can be considered the last level of chronic liver diseases, which go from fibrosis to cirrhosis and to cancer (Santos *et al.*, 2017). This type of cancer has some peculiar epidemiologic characteristics, like differences between geographic

areas, racial and ethnic groups and between males and females (El-Serag & Rudolph, 2007; Ghouri *et al.*, 2017).

The mechanism that leads to HCC is a multistep process that is highly conserved in vertebrates and occurs with higher frequency and severity in males than in females, both in humans and lab animals (Barone *et al.*, 2009; Kim *et al.*, 1991; Li *et al.*, 2017b; Moriya *et al.*, 1998). It is believed that this behaviour can occur due to hormonal and genetic factors or can depend on a higher exposure of males to environmental chemicals and alcohol (Elba *et al.*, 2002). HCC growth period goes from 1 to 20 months and the survival rate depends on the severity of the disease. Since the diagnosis is commonly late, the prognosis for HCC patients is bad (Elba *et al.*, 2002). In the last decades, the frequency and severity of HCC have increased in developed countries, it is estimated that a number between 500 000 and 1 000 000 of new cases of HCC are reported every year, and some authors refer that the aetiology of many HCC cases is multifactorial, including infectious diseases (hepatitis B and hepatitis C viruses), comorbid conditions, exposure to environmental chemicals, liver cirrhosis due to alcohol abuse, diabetes, obesity, intake of food contaminated with aflatoxin, some genetic diseases and the abuse of pharmaceuticals, like paracetamol (Nakra *et al.*, 1973; De Maria *et al.*, 2002; Llovet *et al.*, 2003; Montella *et al.*, 2011; Montella *et al.*, 2015).

1.1.1. Oncogenes and proto-oncogenes

There are two classes of genes that have an essential role in carcinogenesis, oncogenes and tumour suppressor genes. Oncogenes include genes that have the potential to originate a cancer and to modulate positively the cell growth and proliferation. By contrast, tumour suppressors genes protect a cell from a specific step that leads to cancer, negatively regulate cell growth and if there is a mutation in these genes their function is affected, and the cell can progress to cancer. Some authors advocate that the loss of tumour suppressors function is worse than the activation of oncogenes (den Hertog, 2016; Van Beneden *et al.*, 1990).

A proto-oncogene is a normal gene that is transformed into an oncogene due to a mutation or DNA rearrangements and that can lead to overexpression of normal proteins or to altered proteins' production (Lodish *et al.*, 2000).

There is immense information about the molecular basis in mammalian tumours, but not about tumour progression in lower vertebrates and tumour suppressor genes in fish (Van Beneden, Henderson, Blair, Papas, & Gardner, 1990). More than

200 oncogenes and approximately 30 tumour suppressor genes are already identified in mammals, but only a small number was recognized in teleost fish and linked with fish tumours (Baumann & Okihiro, 2000). It is believed that the first "oncogene" was described in a fish, swordtail-platyfish, in 1928 and after that, murine sarcoma virus (*ras*), myc proto-oncogene BHLH transcription factor (*myc*), proto-oncogene tyrosine-protein kinase (*src*), receptor tyrosine kinases (*erb-B*), FBJ murine osteosarcoma viral oncogene homolog (*fos*) and receptor tyrosine kinase Xmrk (*xmrk*) were identified in fish species (Baumann & Okihiro, 2000). The first tumour suppressor gene identified was the retinoblastoma susceptibility gene (*rb1)* and cellular tumour antigen p53 (*p53*), Wilms tumour protein (*wt-1*), von Hippel-Lindau tumour suppressor (*vhl*) and E2F Transcription factor (*e2f*) are some others tumour suppressors already found in fishes (Baumann & Okihiro, 2000). Several types of tumours, like hepatocellular adenomas and carcinomas, are affected by the deregulation of these genes' expression, acting as regulators of cell cycle, proliferation and malignant changings. Those genes are highly involved both in fishes and humans, and several studies focused on the comparison between the zebrafish and human genomes demonstrate that there is a high conservation of tumour suppressors and oncogenes and that the genetic pathways that lead to cancer are evolutionarily conserved, which means that fishes can be used to indirectly study human genes and tumourigenesis (Etchin *et al.*, 2011; Howe *et al.*, 2013; *Chen et al.*, 2014).

1.1.2. Zebrafish as a model organism in cancer research

During their life history, humans and animals are frequently exposed to many different chemicals and compounds that are classified as carcinogens. So, it is of unquestionable importance to understand the link between these chemicals and the appearance of tumours. To do so, scientific researchers have been using animal models, mainly those that are somehow similar to humans, allowing the investigation of mechanisms, development, and progression of diseases and test of new treatments in animals before applying to humans (Shive, 2013).

Teleost fish constitute the largest and most diverse class of vertebrates, with over 30,000 known species (Ravi & Venkatesh, 2018). Their diversity and place in the phylogenetic tree make them ideal subjects for comparative carcinogenesis studies

which may allow more insight into basic mechanisms than studies limited to mammalian models alone (Van Beneden *et al.*, 1990).

Zebrafish (*Danio rerio*) is a species of freshwater environments, originally from Southeast Asia and that has emerged as a model in biological research and as a powerful tool, offering several advantages over traditional lab models, like short life cycle, small size, external embryonic development, high fecundity, lower maintenance costs and the major organs can be easily examined (Bopp *et al.*, 2007; Letrado *et al.*, 2018). In addition to these features, zebrafish also shares tumour suppressors and oncogenes with humans, it has a very low incidence of spontaneous cancers, high rate of tumorigenesis after carcinogens' exposure and is amenable to reverse genetics, allowing in depth studies of tumour suppressors, and therefore being an excellent model for cancer research (Lam & Gong, 2006; den Hertog, 2016).

In ecotoxicological studies, this model is useful to evaluate the effects of inumerous chemicals in many functional aspects, like behaviour and reproduction (Dubińska-Magiera *et al.*, 2016). Several models of zebrafish cancers have been produced by different mechanisms, from chemical carcinogenesis to transgenic models and xenotransplantation in embryos (Shive, 2013). Many chemicals considered carcinogens have been investigated in fish species, like zebrafish, in order to assess their efficacy in fish, to model human carcinogen exposure, and to evaluate environmental toxins (Shive, 2013). Many teleost fish species, and zebrafish in particular, are susceptible to the same carcinogens that affect mammals and produce many neoplasm types in various tissues showing remarkable histopathologic resemblance to human and mammalian cancers (Baumann & Okihiro, 2000; Lam & Gong, 2006).

1.1.3. Carcinogenic agents that induce HCC

Chemical compounds can be divided into five groups, according to the International Agency for Research on Cancer (IARC) identification: 1 – carcinogenic; 2A – probably carcinogenic; 2B – possibly carcinogenic; 3 – not classifiable as to its carcinogenicity; 4 – probably not carcinogenic. Our society commonly uses chemical compounds to satisfy our daily needs in several areas, like food production, agriculture and industry, and, the major part of these compounds is considered carcinogenic, and so capable of inducing carcinogenesis after long exposure. HCC can be easily induced in fish by the same carcinogenic agents that elicit its appearance in mammals (Newell *et al.*, 2008; Tchounwou *et al.*, 2012).

N-nitroso compounds have mutagenic and carcinogenic activities and have been highly used in several studies related to cancer, because they can be found in daily products, like foods, beverages, and drinking water. Diethylnitrosamine (DEN), as others N-nitroso compounds is classified in group 2 by the IARC, because it is capable of inducing disturbance in the nuclear enzymes involved in DNA repair and replication and has a great potential to induce hepatic neoplasms in several fish species (Bunton, 1990; Spitsbergen *et al.*, 2000).

There are many studies focused on DEN impacts, and it is already established that *in vivo* 24h exposure of medaka fish (*Oryzias latipes*), lead to hepatocellular neoplasms and lesions similar to those found in fishes with long-term exposure (Hinton *et al.*, 1988; Bunton, 1990).

Some pharmaceutical compounds, like conazole derivatives, antifungal agents, are also considered carcinogenic agents because they are capable of inducing liver tumours, changes on liver enzymes and hepatic cell proliferation. When exposed to propiconazole, medaka fish registered impacts on hepatocarcinogenesis, that was significantly increased (Tu *et al.*, 2016; van der Laan *et al.*, 2016).

Dimethylbenzanthracene (DMBA) is a hydrocarbon that can be found in the air and tobacco smoke and is commonly used in laboratory to induce neoplasms (Lee *et al.*, 2002; Liu *et al.*, 2015). When zebrafish is exposed to this compound, it is reported the occurrence of hepatic tumours and histological changes similar to those in human neoplasms, like cell proliferation and unusual nuclear morphology (Huang *et al.*, 2012; Santhakumar *et al.*, 2012).

1.2. Non-classical hormonal carcinogenic agents

The liver is a vital organ with a wide array of functions and that is highly sensitive to hormones, such as oestrogens and androgens (Sukocheva, 2018). Several studies using animal models have been suggesting a connection between sex hormones and HCC occurrence, for example supporting that oestradiol and testosterone may have inducer and promoter roles in the all process of hepatocarcinogenesis (Shimizu *et al.*, 1998; Di Maio *et al.*, 2006).

It is well established that males are more susceptible to hepatocarcinogenesis, developing more liver tumours than females and it is believed that this difference occurs due to the higher body mass index and higher levels of androgenic hormones (De Maria *et al.*, 2002; El-Serag & Rudolph, 2007). Androgens are commonly linked with hepatic tumours and produce effects by activating specific hormone receptors present in hepatic cells, but in individuals with HCC, even if chemically induced, these receptors have higher expression and activation (Andrisani *et al.*, 2011). The presence of androgen receptors is associated to a higher risk of tumour occurrence and to a lower survival rate (Ohnishi *et al.*, 1986). It is already proved that tumour growth is inhibited if an anti-androgen drug is used, since it inactivates the androgen receptors (Vesselinovitch *et al.*, 1980).
A normal liver also has oestrogen receptors, that play an essential role in the control of cell proliferation (Hua *et al.*, 2018). Anti-estrogenic drugs reduce nuclear oestrogen receptor activity and inhibit cell proliferation (Dalvai & Bystricky, 2010). Some studies show that oestrogens induce hepatocarcinogenesis and production of DNA adducts that are potentially mutagenic (Yager & Yager, 1980).

Despite the continued evidences supporting a protective role of oestrogens against human HCC there are also supporting that both androgens and oestrogens affect hepatic cell proliferation and promote liver tumours growth (De Maria *et al.*, 2002; Sukocheva, 2018). Recently, exposure of larval and adult zebrafish to 17β-estradiol increased the proliferation of hepatocytes and both the liver volume and mass (Chaturantabut *et al.*, 2019).

1.3. Interrelationship between HCC and lipid metabolism

The liver is essential to lipid, glucose and cholesterol metabolism (Bechmann *et al.*, 2012). It is widely known and established that abnormal lipid metabolism is a hallmark of HCC, but the understanding of lipid metabolism disruption at very early stages of carcinogenesis and the effects of tumour pathways on lipid metabolism are still poorly understood (Long *et al.*, 2018; Pope *et al.*, 2019). The aspects previously referred are critical to understand in the context of HCC initiation in larval stages of oviviparous and oviparous species. This is because their development, growth and survival are highly dependent on yolk mobilization (Nakagawa *et al.*, 2018; Sant & Timme-Laragy, 2018; Piccinin *et al.*, 2019).
Increasing studies show that changes in lipid metabolism are essential for tumour growth, survival and proliferation, since they support cancer cells with bigger energy production, macromolecular biosynthesis and preservation of the redox balance (De Matteis *et al.*, 2018; Yin *et al.*, 2017). Therefore, HCC has some lipid metabolic alterations that could be used as therapeutic targets, for example it is known that fatty acids biosynthesis is commonly enhanced in HCC (Wang *et al.*, 2016; Pope *et al.*, 2019).

Under normal circumstances, the liver produces, stores and releases glucose depending on the body's needs. After an individual takes a meal, blood glucose enters his hepatocytes and is converted into pyruvate and oxidized by Krebs cycle and oxidative phosphorylation (figure 1). Or it can be used in fatty acid synthesis pathway through *de novo* lipogenesis (DNL). If anything is wrong with the liver, glucose metabolism can be changed. In HCC, there is a high glucose metabolism, which is essential for the proliferative rate of the tumour, and for its growth and survival. So, diabetic patients, that commonly have higher levels of glucose, can have higher rates of tumourigenesis (De Matteis *et al.*, 2018).

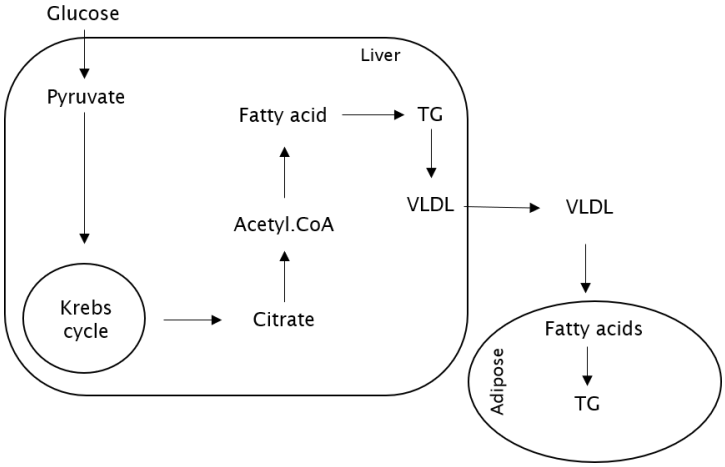


Figure 1. Classical representation of lipid metabolism. TG – triglycerides; VLDL - very low-density lipoprotein. Adapted from Lu & Hooi, (2017).

DNL and lipogenesis are commonly increased in tumours, allowing the presence of extra lipids and lipid precursors during the rapid cell proliferation that occurs. Some enzymes are identified as promoters of DNL, including *fatty acid synthase (fasn), stearoyl-CoA desaturase (scd)* and *acetyl-CoA carboxylase (acc)* (Yin *et al.*, 2017).

The incidence of HCC in developed countries is increasing rapidly, and a big part of these patients are obese and display symptoms of metabolic syndrome and non-alcoholic steatohepatitis (NASH), that includes liver steatosis, hepatocellular injury, fibrosis and inflammations (Sarwar *et al.*, 2018). It is not clearly understood how obesity influences hepatocarcinogenesis, but some factors like elevated proinflammatory cytokines induced by oxidative stress, adipokines' dysregulation and altered gut microbiota are commonly believed to promote HCC development (Nakagawa *et al.*, 2018).

Several studies have been focused in lipid metabolic reprogramming in carcinogenesis (Beloribi-Djefaflia *et al.*, 2016; Cheng *et al.*, 2018; Nakagawa *et al.*, 2018). Fatty acids can act as signalling molecules, storage compounds and energy sources, that are essential for cancer cells proliferation (Currie *et al.*, 2013). The lipid uptake and fatty acids β-oxidation are highly altered in several types of cancer, suggesting the occurrence of a metabolic flexibility and a connection with metabolic pathways that are altered by tumour microenvironment (Currie *et al.*, 2013; Boroughs & DeBerardinis, 2015; Long *et al.*, 2018). Therefore, enzymes involved in fatty acids pathways deserve attention as they may be used as therapeutic targets. It has been established that genes involved in fatty acids

biosynthesis are upregulated in HCC tissues when compared to normal liver tissues. Additionally, the Wnt/β-catenin pathway is highly conserved in vertebrates and it is activated in more than 50% of HCC cases and it is described that this activation represses lipogenesis in white adipose tissue, and the transcriptional targets, like *myc,* of the Wnt pathway are commonly altered and can be considered oncogenic drivers in hepatocellular carcinoma (Shang *et al.*, 2017; De Matteis *et al.*, 2018; Khalaf *et al.*, 2018; Yao *et al.*, 2018).

1.4. Aims of the work and rational of the targeted genes

From the expressed in the above sections, it becomes clear that numerous compounds are able to induce and model the progression of HCC, from carcinogenic agents, like DEN, to non-carcinogenic chemicals, like lipid disruptors and sex hormones. This happens not only in humans, but also in rodents and, several fish species, especially medaka and zebrafish, are among the most used model organisms for cancer studies, including on chemical carcinogenesis.

Despite everything known about the aetiology and treatment of HCC, there is a question that remains unanswered: what is the role of androgens and oestrogens in initiation and progression of this type of cancer? Li *et al.*, (2017) refers that sex hormones act differently in tumour progression. It is already quite established that androgens have a promotive role, which means that the activation of their receptors lead to a higher expression of genes commonly associated to cell cycle progression and also lead to a lower expression of genes that are liver-specific, changing its normal function (Andrisani *et al.*, 2011). Males, not only have higher susceptibility to develop a HCC, but when they develop the tumour, they have lower survival rates and poorer prognosis (El-Serag & Rudolph, 2007). There are on-going discussions, and even contradictory epidemiological and experimental data about the roles of sex hormones signalling and the onset and progression of tumorigenesis. On the other hand, oestrogens' role on HCC is not yet clear, and there is no consensus between authors, since some studies show that oestrogens act as promotors – just like androgens -, and others declare that these hormones have the opposite role, acting against the development of a cancer (Rice & Whitehead, 2006; Ferretti *et al.*, 2007).

Bearing in mind the connection that exists between HCC and lipid pathways, this study uses chemical induced HCC to study and help clarifying the protective or

promotor roles of oestrogens and androgens when triggering tumour processes, and their effects on lipid-related genes regulation.

To achieve this, zebrafish larvae were exposed to DEN, and inducer of liver tumour, to DEN plus testosterone and DEN plus ethinylestradiol, to understand the effect of sex hormones. Then, biometric, histological and molecular procedures were performed. Biometric and histologic analysis were used to evaluate possible morphological differences arising from the exposure to DEN, since it is suggested by some studies that the exposure to some chemicals causes stress that affects the morphology of the exposed individuals (Lammer *et al.*, 2009; Li *et al.*, 2017a). Moreover, if there would be impacts on lipid metabolism, then the growth of larvae could be impacted, because it depends on the mobilization of yolk lipids.

As to the molecular approach, four genes related to carcinogenesis initiation and four genes related to lipid metabolism, all explained below, were chosen and assessed by RT-qPCR.

The genes *myca* and *mycb* are homologous genes for c-*myc* (*myc proto-oncogene BHLH transcription factor*), take part in Wnt signalling pathway and regulate cell growth and proliferation, playing an essential role in the genesis of a wide variety of tumours (Corral *et al.*, 1988; Pelengaris & Khan, 2003). These genes are highly expressed in some tumours, like HCC, breast and prostate carcinomas, and an altered number of *myc* copies were detected in early human gastric cancer, suggesting the involvement of *myc* genes in the initiation of this tumour (Thorgeirsson & Grisham, 2002; Takahashi *et al.*, 2007; Calcagno *et al.*, 2008; Chen & Olopade, 2008; Costa Raiol *et al.*, 2008). Corral *et al.* (1988) reports a higher expression of *myc* genes as soon as one month after the exposure of rats to DEN. In the major part of experimental models of induced tumorigenesis, *myc* activity is important to maintain tumour cells, and its down-regulation leads to apoptosis or senescence (Arvanitis & Felsher, 2006). Kaposi-Novak *et al.* (2009) identified *myc* as a driver gene and a regulator of malignant transformation in hepatocarcinogenesis initiation, and Li *et al.* (2013), after exposing zebrafish to doxycycline, reported an increase in *myc* expression.

The gene *mapk1* (*mitogen-activated protein kinase 1*) is also involved in cell proliferation, differentiation and transcription regulation. Zebrafish exposed to DMBA during 24h have an increased expression of *mapk1*, and this gene's expression is upregulated in 50-58% of HCC cases (Ito *et al.*, 1998; Hoffmann *et*

al., 2011; Mirbahai *et al.*, 2011). This gene belongs to the MAPK/ERK signalling pathway, that can be activated due to extracellular signals, such as growth factors, tumour-promoting substances or hormones, whose activation is related to malignant alterations in hepatocytes and tumour development in HCC (Yang & Liu, 2017).

The role of *p21* (*cyclin-dependent kinase inhibitor 1*) is intimately related with its subcellular location. It is a well-known tumour suppressor, but in some conditions, it promotes tumour growth. *p21* located in nucleus can inhibit cell proliferation and act as a proapoptotic gene, but its cytoplasmic form can have oncogenic and anti-apoptotic activities (Zhang *et al.*, 2009; Ohkoshi *et al.*, 2015). So it is involved in cell cycle regulation and some studies found that *p21* is down-regulated in HCC tissues, demonstrating its tumour suppressive function (Ohkoshi *et al.*, 2015). In the absence of *p21*, proliferation of cells with DNA damages result in rapid cancer formation (Willenbring *et al.*, 2008).

p21 gene is a key player in three p53 pathways that are involved in cell cycle regulation and apoptosis. Other studies suggest that this gene is up-regulated in HCC tissues and that it can play an important role in tumour progression by inactivating some pathways (Zhang *et al.*, 2009).

The gene *lipca* (*lipase hepatic a*) is involved in lipid mobilization and catalyses the hydrolysis of triglycerides (figure 2). The *scd* (*stearoyl-CoA desaturase*) is crucial for fatty acid metabolism and catalyses the rate-limiting step in the formation of monounsaturated fatty acids (figure 3). The *acaa2* (*acetyl-CoA acyltransferase 2* or *3-ketoacyl-CoA thiolase*) catalyses the last phase of fatty acid β-oxidation to release acetyl CoA for Krebs cycle (figure 4). Finally, the *elovl1a* (*fatty acids chain elongase 1*) catalyses the synthesis of monounsaturated long chain fatty acids (figure 5). All these genes were assessed in a study where zebrafish larvae were exposed to doxycycline (a tetracycline antibiotic) for 72h and it is reported that *elovl1a* is the only gene whose expression suffered down-regulation, while the others' expression was up-regulated (Yao *et al.*, 2018).

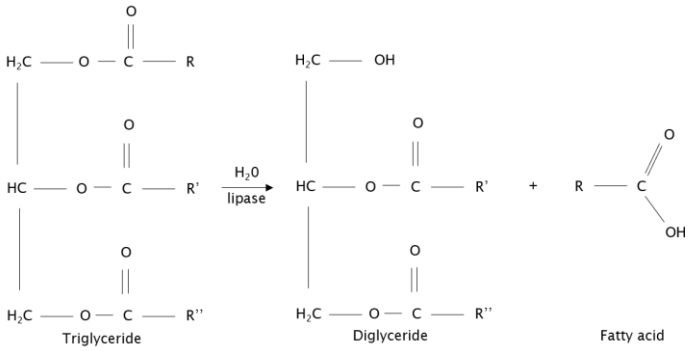


Figure 2. Hydrolysis of triglycerides by the action of hepatic lipase (gene *lipca*).

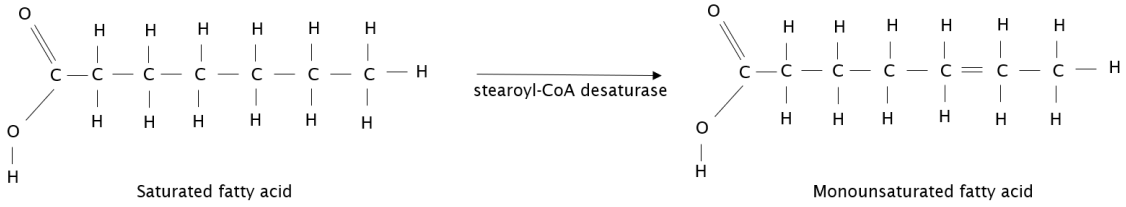


Figure 3. Monounsaturated fatty acid formation by the action of stearoyl-CoA desaturase (gene *scd*).

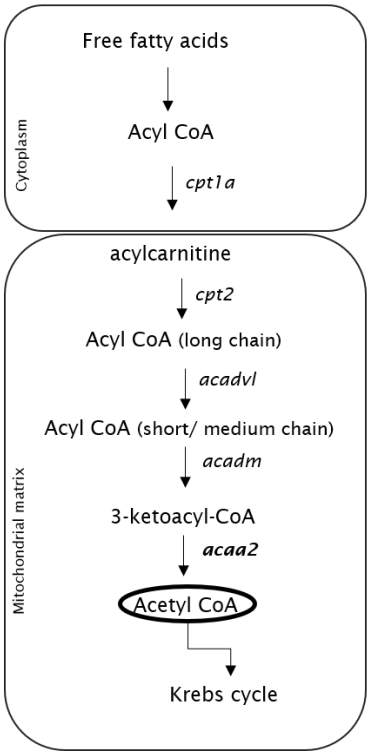


Figure 4. Fatty acid β-oxidation. *cpt1a* – carnitine palmitoyl-transferase 1a; *cpt2* – carnitine palmitoyl-transferase 2; *acadvl* – very long-chain acyl-CoA dehydrogenase; *acadm* – medium chain acyl-CoA dehydrogenase; *acaa2* – acetyl-CoA acyltransferase 2. Adapted from Soni *et al.*, (2016).

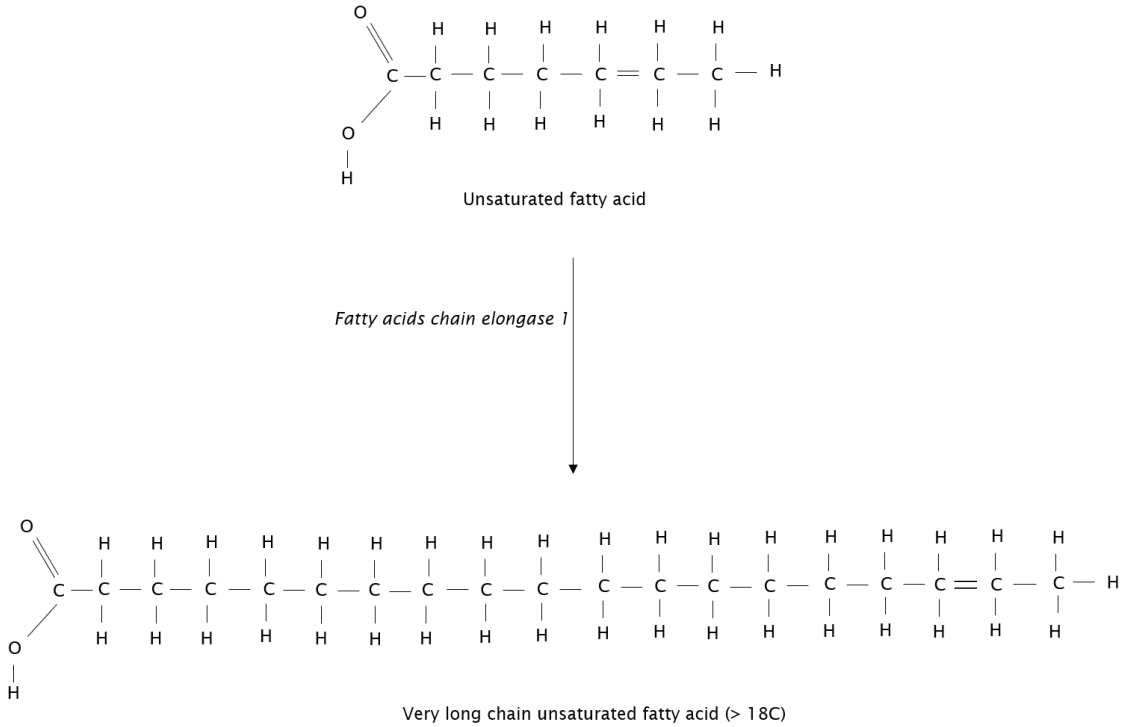


Figure 5. Synthesis of very long chain monounsaturated fatty acids, fatty acids with more than 18C, by the action of fatty acids chain elongase 1 (gene *elovl1a*).

Considering the high homology that exists between zebrafish and mammalian class, approximately 70%, it is intended that the results obtained with the former can be translated to other living organisms, especially to humans, and lead to more in-depth fundamental background knowledge that will, hopefully, be translated in a more efficient risk identification, risk management, prevention and treatment.

2. Materials & Methods

2.1. Animal breeding and exposure

Forty-six adult zebrafish were available to breed and were maintained in two aquariums of 30L with continuous aeration and filtering (biological and mechanical). Additionally, 60% of the water was changed twice a week. The water temperature was kept at 28ºC. The fishes were fed every morning with artemia (instant baby shrine – Ocean Nutrition, Belgium) and in the afternoon with TetraMin flakes (Tetra, Germany). The water pH, ammonia and nitrites levels, dissolved O_2, salinity and conductivity, were regularly measured to guarantee adequate conditions to the fishes.

Table 1. Experimental design of animal breeding and exposure.

-1	0 dpf	1 dpf	2 dpf	3 dpf	4 dpf	5 dpf
Mating and breed	Spawning	-	-	Beginning of the exposure	24h of exposure, renewal of the solutions	End of the exposure

For offspring production, two males for each female were kept separated and then put together and incited to mating and breed after a sudden onset of light early in the morning. Eggs were collected approximately 1h later and, subsequently rinsed several times with preheated, aerated deionised water, as described in Madureira *et al.* (2011). Embryos of three days old were used for starting exposures, as described below.

Groups of embryos were exposed either to solvent or to the carcinogen diethylnitrosamine (N-nitrosodiethylamine; DEN; CASRN 55-18-5; Sigma-Aldrich), alone or mixed with an androgen (testosterone, CASRN 58-22-0; Sigma-Aldrich) or an oestrogen (ethinylestradiol, CASRN 57-63-6; Sigma-Aldrich). Accordingly, one group was exposed solely to the solvent ethanol (at 0.01%). Other group was subjected to DEN only (at 350 mg/L), another to a mixture of DEN (at 350 mg/L) and testosterone (at 15 ng/L) (TEST), and another to a mixture of DEN and ethinylestradiol (at 15 ng/L) (EE2). Each exposure was made in triplicate, starting with 200 fertilized eggs immersed in 200 ml of solution. Laboratory beakers were used as exposure tanks. After 24h of exposure, the solutions were gently renewed.

The exposure lasted for 48h. Larvae were euthanized by an overdose of 2-phenoxyethanol (CASRN 122-99-6, Merck).

2.2. Biometric parameters

To evaluate eventual morphological and phenotypical differences between treatment groups, and using an image of each larvae, seven points were selected as established by Li *et al.* (2017): (1) mouth, (2) frontal concave of pericardium, (3) posterior concave of pericardium, (4) concave of yolk sac, (5) tail, (6) first fishbone point, (7) salient point of head (figure 6). Straight lines between each pair of points were measured using the ImageJ software (available at https://imagej.nih.gov/ij/), resulting in a total of 21 lines for each image: 1,2; 1,3; 1,4; 1,5; 1,6; 1,7; 2,3; 2,4; 2,5; 2,6; 2,7; 3,4; 3,5; 3,6; 3,7; 4,5; 4,6; 4,7; 5,6; 5,7; 6,7. These photos were taken with a SZX10 stereomicroscope (Olympus, Denmark), coupled with a DP21 digital camera (Olympus, Denmark). A total of 34 photographs were taken for each treatment group.

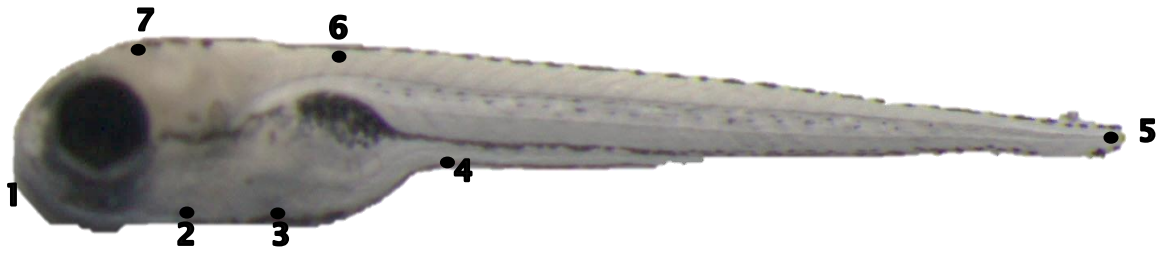


Figure 6. Localization of the seven selected points in the larvae. (1) mouth, (2) frontal concave of pericardium, (3) posterior concave of pericardium, (4) concave of yolk sac, (5) extremity of the tail, (6) first fishbone point, (7) salient point of head.

2.3. Histological procedures

The euthanized larvae were fixed in 10% buffered formalin and stored at room temperature until further processing for histological studies.

The post-fixation process followed routine steps: dehydration (in ethanol), clearing (in xylene) and embedding (either in paraffin or in a methacrylate-based plastic resin).

The processing for paraffin was done using an automatic tissue processor (Leica TP1020, Germany) according to the protocol in Appendix I. A workstation (Leica EG1140H, Germany) was used for preparing the final paraffin blocks. As a final step they were put on an iced plat so that the paraffin turns solid and make them ready to be sectioned. Sections of 3 μm were obtained using a fully automatic microtome (Leica RM2155, Germany) with disposable steel knives and the section extension was performed in a hot water bath (70ºC). After staining the slides with haematoxylin and eosin (Appendix II), they were mounted with Coverquick 2000 (VWR, France) and analysed in optical microscope.

As to the processing for methacrylate embedding, samples were dehydrated (in ethanol in different concentrations), and then subjected to pre-infiltration and infiltration steps, as described in Appendix III.
Once the support blocks were ready, samples were sectioned, also at 3 μm in thickness, but using a tungsten-carbide knife and the aforementioned microtome. Section extension was performed in a warm water bath (20-25ºC). Staining was performed with haematoxylin and eosin (Appendix IV). After final mounting as described for paraffin, sections were used in a stereological study.

2.4. Stereological analysis

The total volume of the liver was estimated by the so-called "Principle of Cavalieri" (1635), as implemented in stereology (Gundersen & Jensen, 1987). This analysis required the measurement of the areas of the liver as seen in serial sections of the organ, in each animal. To do so, nine animals (three from each replicate) per treatment were prepared in methacrylate (Technovit Methyl Methacrylate and Glycol Methacrylate, Kulzer, Germany), and serially sectioned. The option for the plastic resin was to avoid the retraction seen in other embedding media, such as paraffin, and thus to produce measurements that are not biased by that artefact. Sections were analysed using the C.A.S.T. Grid Software (Olympus, Denmark), controlling an Olympus BX50 microscope (Olympus, Denmark) coupled to a SONY DXC-107AP camera (Sony, Japan). The areas of the liver, in all sections, were measured semi-automatically by digital delimitation and calculation via the software. The liver volume (V) was estimated using the Cavalieri principle, as:

$$V = \text{Distance between measured surfaces x} \sum \text{Area of liver in the section}$$

where the distance between surfaces is here the nominal section thickness (3 μm).

2.5. RNA extraction and cDNA synthesis

Three pools of ten larvae/replica (n = 9/treatment group) were initially homogenised with a turrax (IKA, Germany), and the total RNA was extracted using a commercial kit GRS Total RNA Kit - Tissue (GRiSP Research Solutions, Portugal), according to manufacturer's recommendations.
RNA concentration and purity (λ 260/280 and 260/230 nm) were measured using a μDrop Plate (Thermo Scientific, USA) and a Multiskan GO equipment (Thermo Scientific, USA) (appendix V). cDNA synthesis was performed with iScript™ Reverse Transcription Supermix for RT-qPCR kit (BioRad), using 500 ng of total RNA for 20 μL of reaction volume, following the manufacturer's protocol (appendix VI).

2.6. Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed with a CFX Connect real-time PCR detection system, using a CFX Manager software (Bio-Rad). SYBR Green reactions always included 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad), 5 μL of cDNA and 200 nM of each primer. No template controls were performed with RNA-free water.
Primer sequences for the target and reference genes have been published elsewhere (details in Table 1), but optimization of annealing temperatures with gradient tests were performed here, using 5 cDNA samples from the distinct treatment groups and a range of temperatures from 55ºC to 60ºC.
Next, calibration curves for each gene were done using seven diluted standards (starting from 1:5) from the same samples used in the gradient tests. The final protocols for each gene are detailed in appendix VII and VIII. The protocol for samples analysis is detailed in appendix IX.
A multiple reference gene selection was made by testing three genes – *tubulin alpha 1* (*tuba1*), β – actin 2 (*β-actin2*) and *elongation factor 1 alpha* (*elfa)*, based on the literature (Vanhauwaert *et al.*, 2014). The best combination of the two most stable reference genes (*elfa* and *β-actin2*) was obtained using NormFinder algorithm (Andersen *et al.*, 2004). Normalization was made with the geometric mean of both genes and calculated according to Pfaffl method (Pfaffl, 2001).

2.7. Statistical analysis

All data were statistically analysed using Past3 software, version 3.25 (available at https://folk.uio.no/ohammer/past/) (Hammer *et al.*, 2001). The graphs were generated using GraphPad Prism 6 software. Normality and homogeneity of variances were tested using the Shapiro-Wilk's W-test and Levene's test, respectively. One-way ANOVA was done followed by Tukey multiple comparison test. If assumptions of normality and homoscedasticity were not met, the non-parametric Kruskal-Wallis test was applied followed by Mann-Witney test with sequential Bonferroni corrections. Significant differences between treatment groups were considered when p-value < 0.05.

Table 2. Primer sequences for target and reference genes.

Gene	Primer Forward	Primer Reverse	Reference
acaa2	GCTCACAAACTCACCCCATT	TCCACCAGGTCCATGTCTTT	(Blanc, 2016)
elovl1a	CTTGCTGGGATACGTCTTCTC	GATGCTGTCAGGTGTCAGAG	(Yin *et al.*, 2011)
lipca	ACTGAGCCTGAAGCCAAGATGAAG	CGTCTACCGACCAGCCATGAATG	(Fetter *et al.*, 2015)
scd	ACCCGGAAGTCATCGAGAGA	GAGGAGCGTCGGGATGAAAT	(Dhanasiri *et al.*, 2013)
mapk1	GAAAAGTTAATTGTTTTTATGGTAT	ATTAACAAACATATATTTTCTACTAATAAT	(Mirbahai *et al.*, 2011)
myca	CGCGCTACGGGATGAGATCCCT	GCAGGGGGTGGGAGTTCTTGGA	(Li *et al.*, 2013)
mycb	AAGCGGCCAAAGTGGTGATCCT	CACTACTTTGCCACACCCTCGC	(Li *et al.*, 2013)
p21	GACCAACATCACAGATTTCTAC	TGTCAATAACGCTGCTACG	(Soares *et al.*, 2012)
tuba1	TCATCTTCTCCTTCCACACT	GTACGTGGGTGAGGGTAT	(Vanhauwaert *et al.*, 2014)
β-actin2	ACGATGGATGGGAAGACA	AAATTGCCGCACTGGTT	(Vanhauwaert *et al.*, 2014)
elfa	GGAGACTGGTGTCCTCAA	GGTGCATCTAACAGACTT	(Vanhauwaert *et al.*, 2014)

3. Results

All data are shown in boxplots, with the identification of the results of statistical comparison. Significant differences between treatment groups were considered when p-value <0.05.

3.1. Fish Biometric Parameters and Histological Images

Figure 7 shows the larva weight, in mg, which did not differ statistically between groups at the p-value <0.05 level [F (3,8) = 1.03, p=0.43].

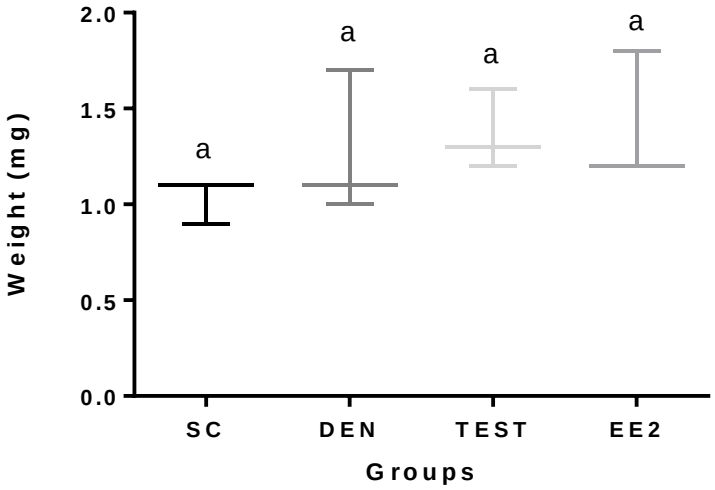


Figure 7. Larvae weight (mg) in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

Figures 8, 9, 10 and 11 show the results of the measurement and analysis of the various types of lengths.

By the analysis of figures 8B, 8E, 9A, 9B, 9E, 10B, 10D, 10E, 11B and 11F it is concluded that there are no differences between the fish group exposed to solvent (SC) and those groups that were exposed to diethylnitrosamine (DEN), to DEN plus testosterone (TEST), and to DEN plus ethinylestradiol (EE2) at p-value <0.05 level [p= (0,12; 0,76)].

In contrast, there are significative differences between the SC group and the others in six parameters (figures 8D, 9D, 10C, 11A, 11D and 11E) at p-value <0.05 level [p = ($8{,}97\times10^{-9}$; $1{,}37\times10^{-4}$)]. It is clear that all the selected measures that include point 5 (extremity of the tail) have mean lower values in all the fish exposed to diethylnitrosamine (DEN, TEST and EE2) when compared to the SC animals. The groups exposed to the steroids did not differ from the DEN only group.

In addition to the differences referred before that involve more than two groups, there are a few more significant ones at p-value <0.05 level involving group pairs. Specifically, SC and DEN groups differ in two length parameters (figures 9C and 10A) with p= 0,018 and p= 0,009, respectively, between TEST e EE2 in one parameter (figure 10C) with p= $1{,}46\times10^{-5}$, and between DEN and TEST in one parameter (figure 8A) with p= 0,013. There is one parameter, showed in figure 8C with p= $1{,}75\times10^{-4}$, in which the fish exposed to the androgen TEST are found to be significantly lengthier than the animals in other groups.

Figure 12 represents the difference between paraffin (A1 and A2) and methacrylate embedding (B1 and B2). By the analysis of images A1 and A2, it is clear that there is no optical definition, there are some parts of the larva that do not appear, and it is difficult to distinguish the different components. Since the methacrylate embedding offered better results, the slides that result from this embedding were used to analyse the liver of the different treatment groups (figure 13). By a qualitative analysis, it seems that there are no big differences between figures 13A, 13C and 13D – Solvent Control, Diethylnitrosamine plus Testosterone and Diethylnitrosamine plus Ethynilestradiol, respectively. On the other hand, figure 13B represents the liver of a larva exposed to diethylnitrosamine only and it seems that the cytoplasm appears more basophilic, which means that the cells are more active, with higher proliferative rate.

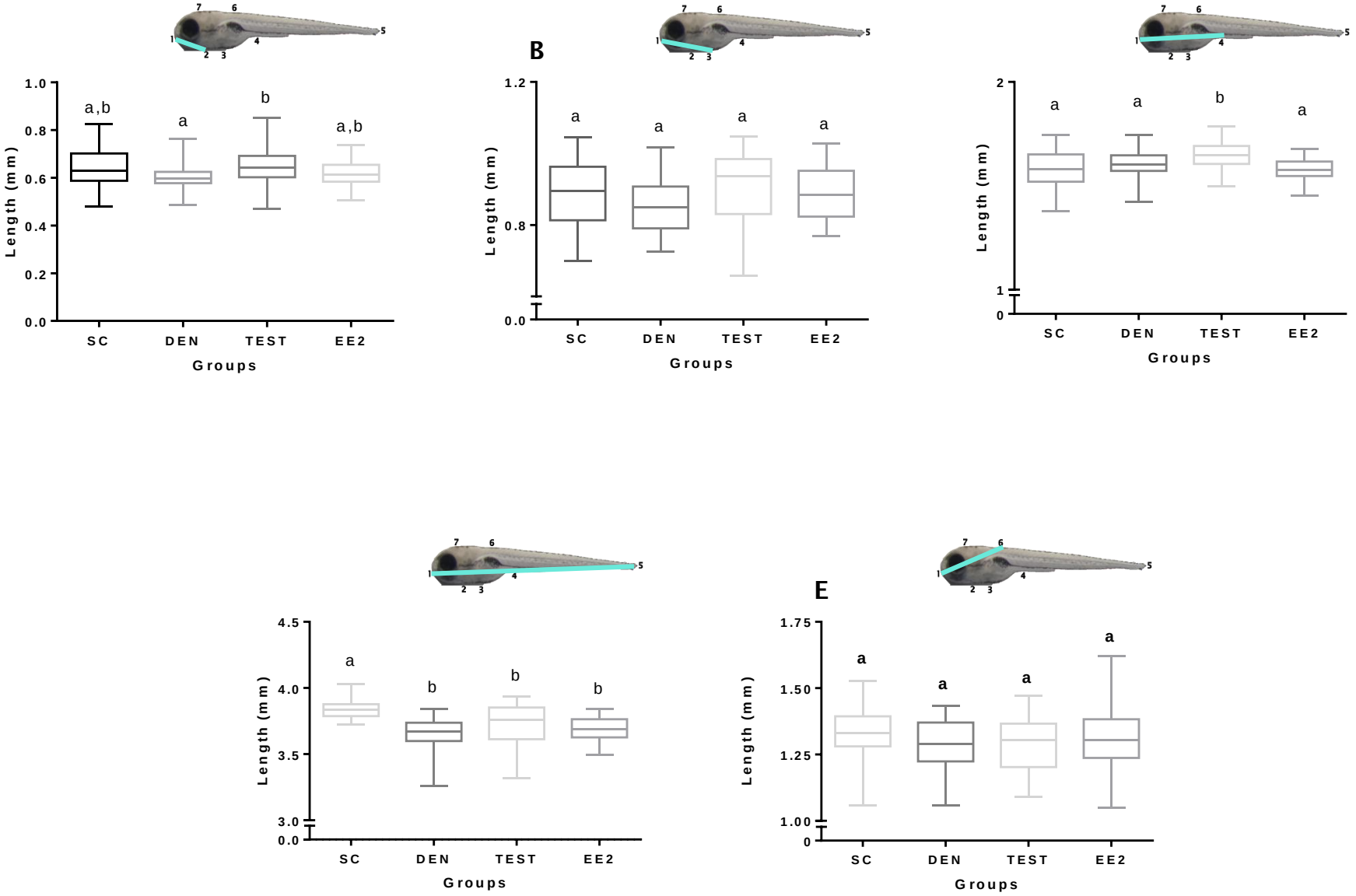


Figure 8. Length between: A - mouth and frontal concave of pericardium (1,2); B – mouth and posterior concave of pericardium (1,3); C – mouth and concave of yolk sac (1,4); D – mouth and tail (1,5); E – mouth and first fishbone point (1,6).

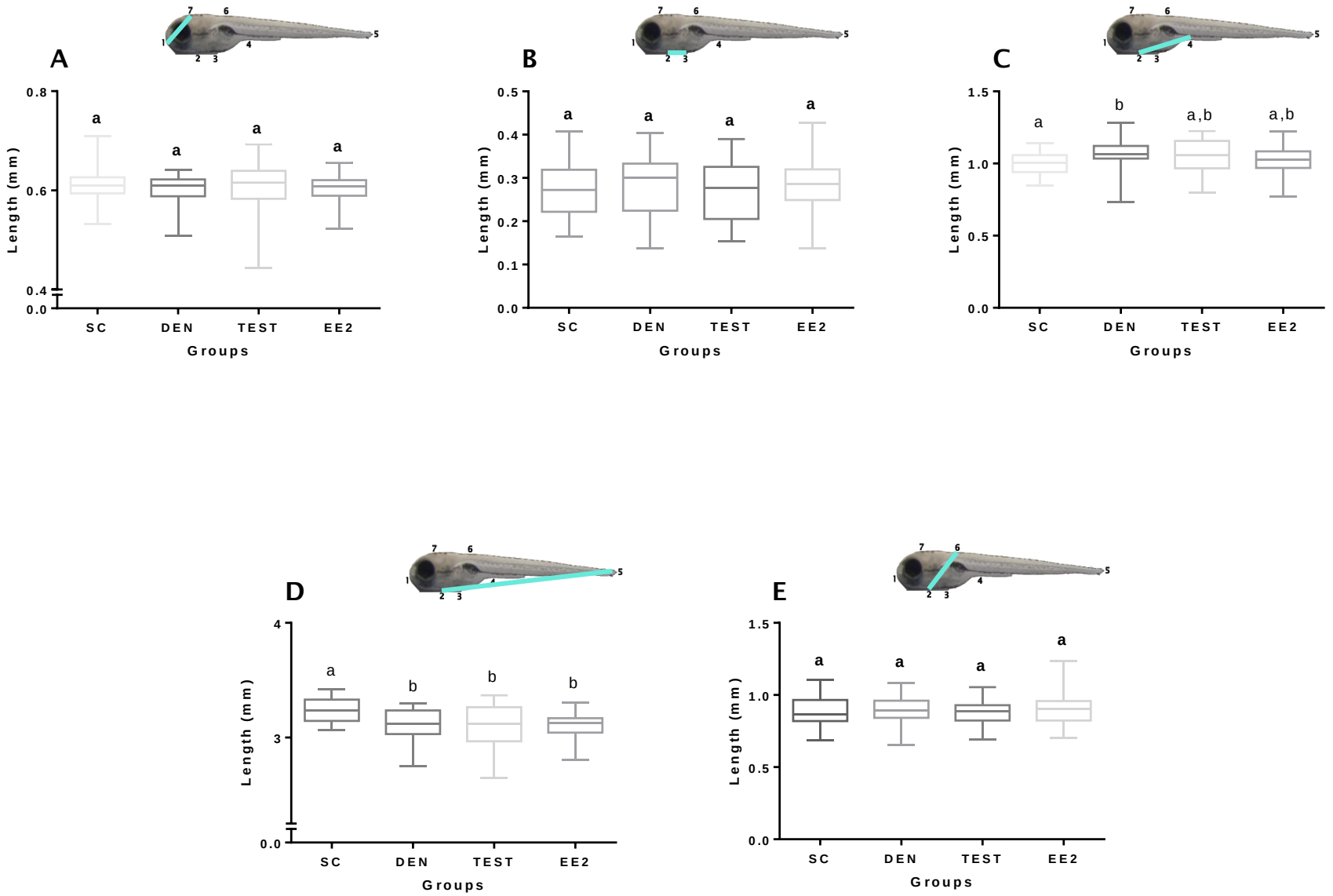


Figure 9. Length between: A – mouth and salient point of head (1,7); B – frontal concave of pericardium and posterior concave of pericardium (2,3); C – frontal concave of pericardium and concave of yolk sac (2,4); D – frontal concave of pericardium and tail (2,5); E – frontal concave of pericardium and first fishbone point (2,6).

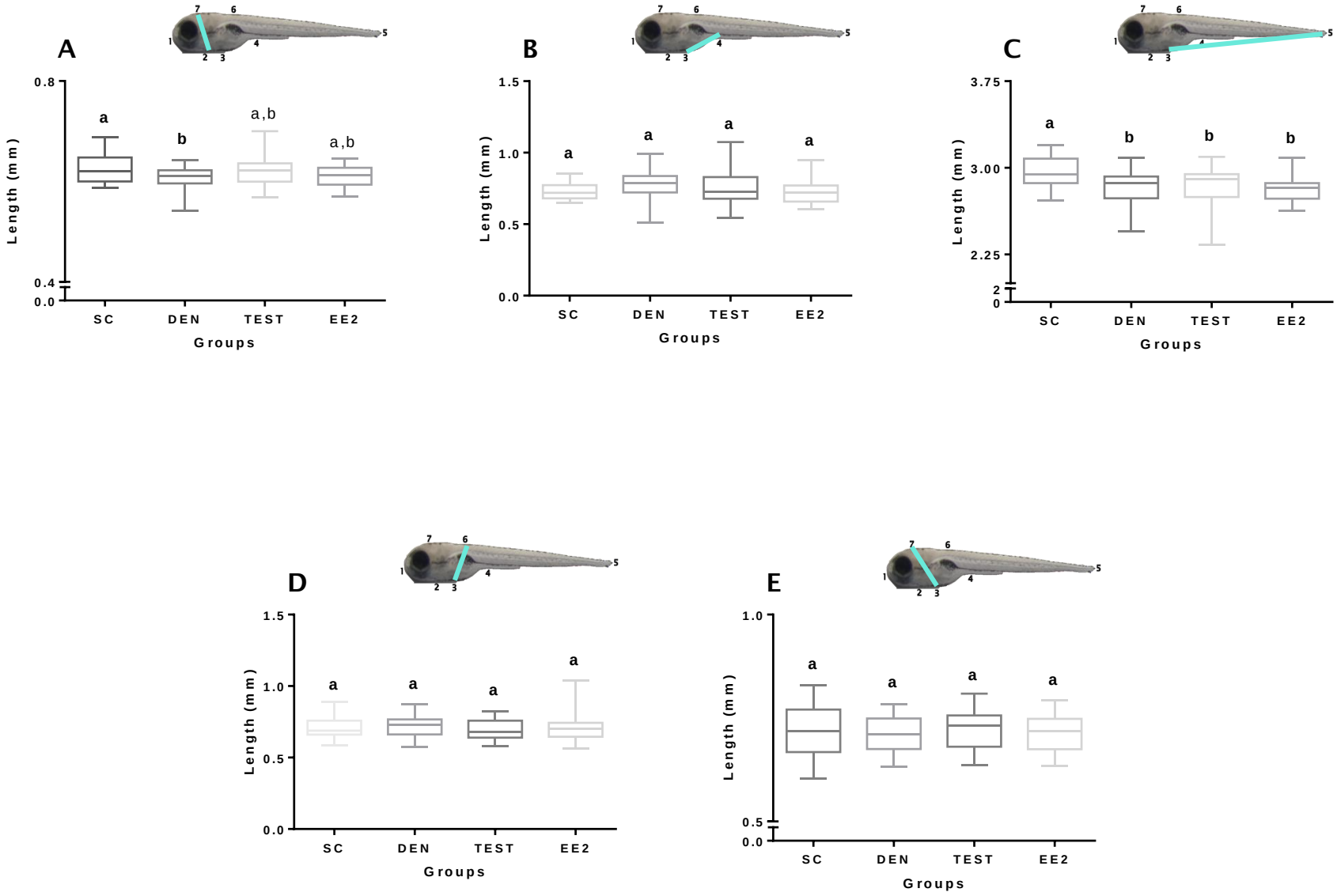


Figure 10. Length between: A - frontal concave of pericardium and salient point of head (2,7); B – posterior concave of pericardium and concave of yolk sac (3,4); C – posterior concave of pericardium and tail (3,5); D – posterior concave of pericardium and first fishbone point (3,6); E – posterior concave of pericardium and salient point of head (3,7).

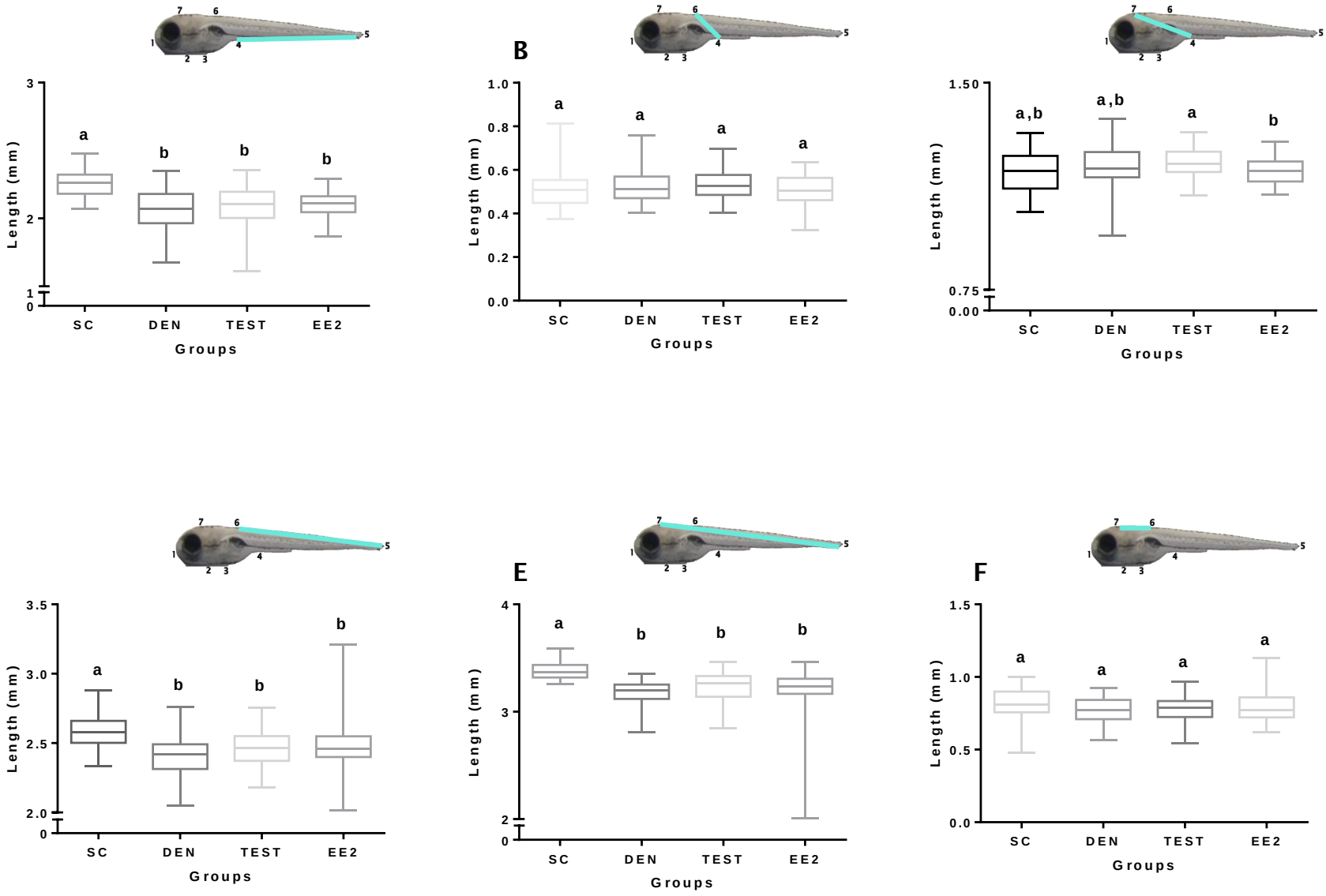


Figure 11. Length between: A – concave of yolk sac and tail (4,5); B – concave of yolk sac and first fishbone point (4,6); C – concave of yolk sac and salient point of head (4,7); D – tail and first fishbone point (5,6); E – tail and salient point of head (5,7); F – first fishbone point and salient point of head (6,7).

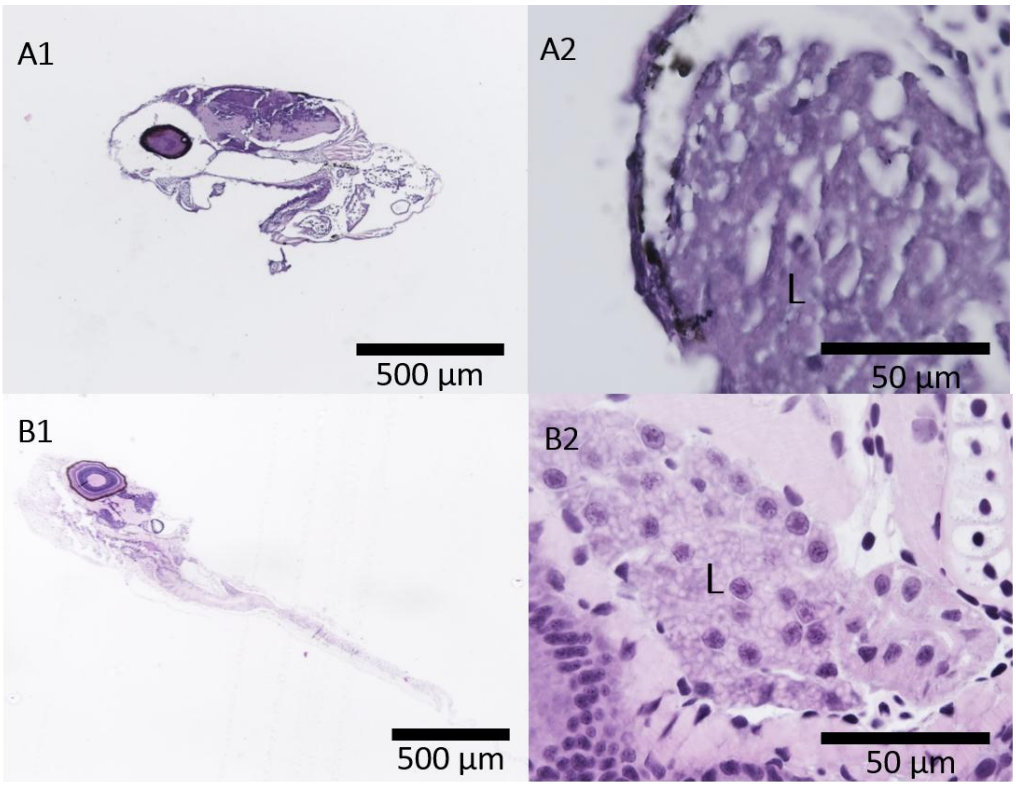


Figure 12. Larva and liver (L) embedded in paraffin (A1 and A2) and larva and liver embedded in methacrylate (B1 and B2).

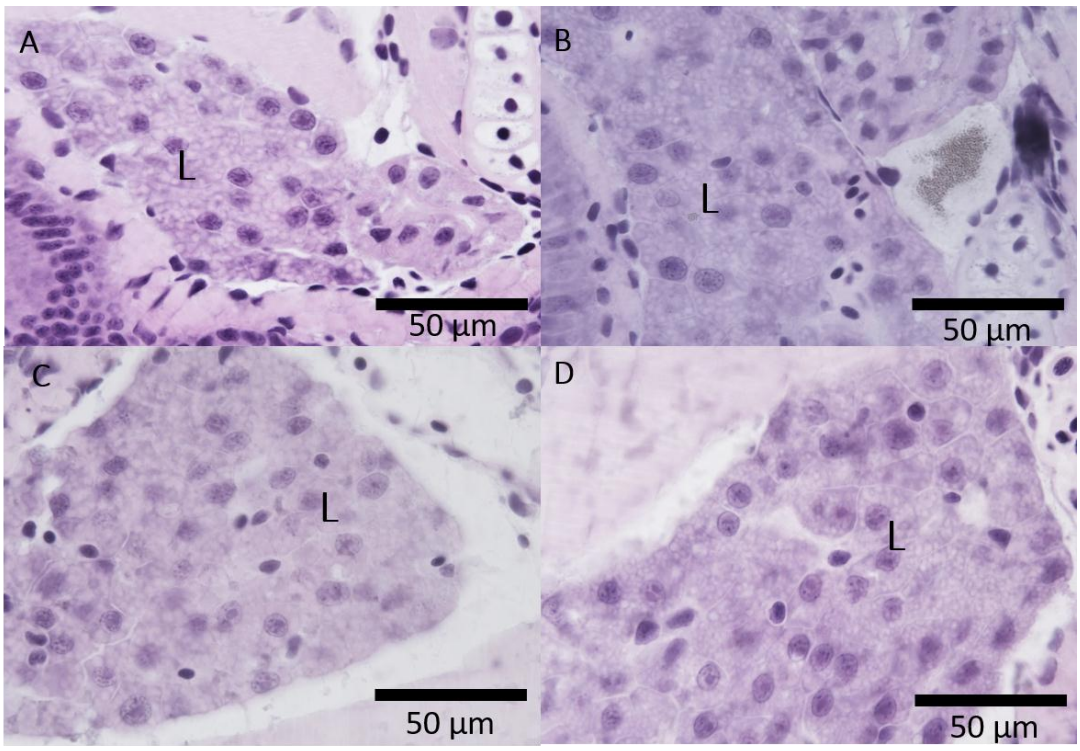


Figure 13. Histological images of the liver (L) of Solvent Control (A), Diethylnitrosamine (B), Diethylnitrosamine plus Testosterone (C) and Diethylnitrosamine plus Ethinylestradiol (D).

3.2. Liver Volume

Liver volume estimations using stereology are represented in figure 12. The DEN group has a significantly bigger volume than the others, and no further significant differences were unveiled at p-value <0.05 level [F (3, 32) = 6,08, p= 0.002].

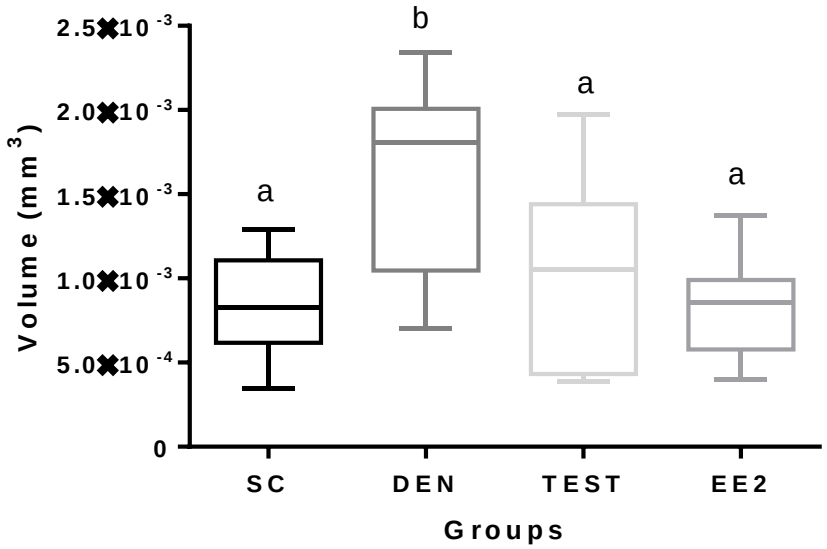


Figure 14. Liver volume (mm^3) in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Tukey test, comparing the experimental conditions.

3.3. Quantification of mRNA

Figures 13 to 20 present the relative mRNA levels (aka gene expression) of the genes *acaa2, elovl1a, lipca, scd, mapk1, myca, mycb* and *p21*.

The *acaa2* (figure 13) is significantly greatly expressed in DEN group and less expressed in SC group, at a p-value <0.05 level [F (3, 32) = 3,71, p= 0,021]. The TEST and EE2 groups show intermediate expression levels, as they did not differ from the other groups.

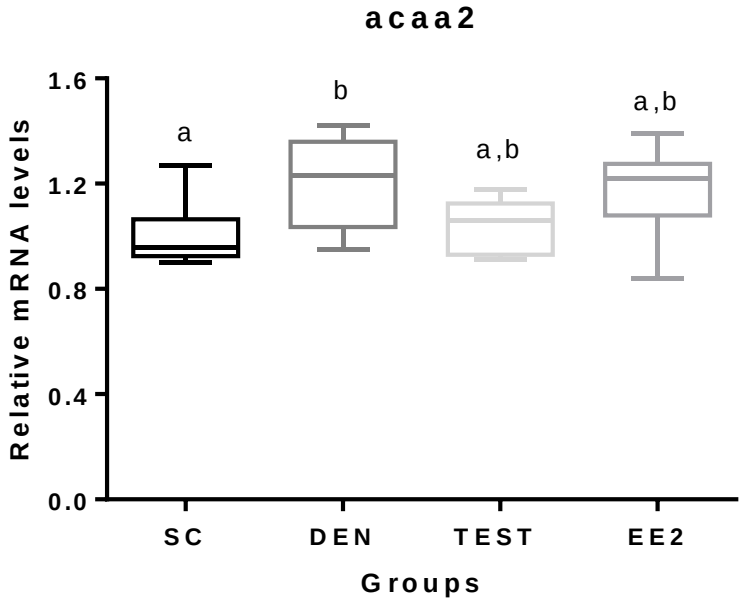


Figure 15. Relative mRNA levels of *acaa2* in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Tukey test, comparing the experimental conditions.

In what concerns to *elovl1a* (figure 14), *lipca* (figure 15), *scd* (figure 16), *mapk1* (figure 17), *myca* (figure 18) and *mycb* (figure 19) there were no significant differences between the experimental groups, at a p-value <0.05 level [p= (0,09; 0,94)].

As to gene *p21* (figure 20), it has a significantly much lower expression in SC group compared with all the other (which not differ between them) at a p-value <0.05 level [F (3, 32) = 25,25, p= $1{,}44 \times 10^{-8}$].

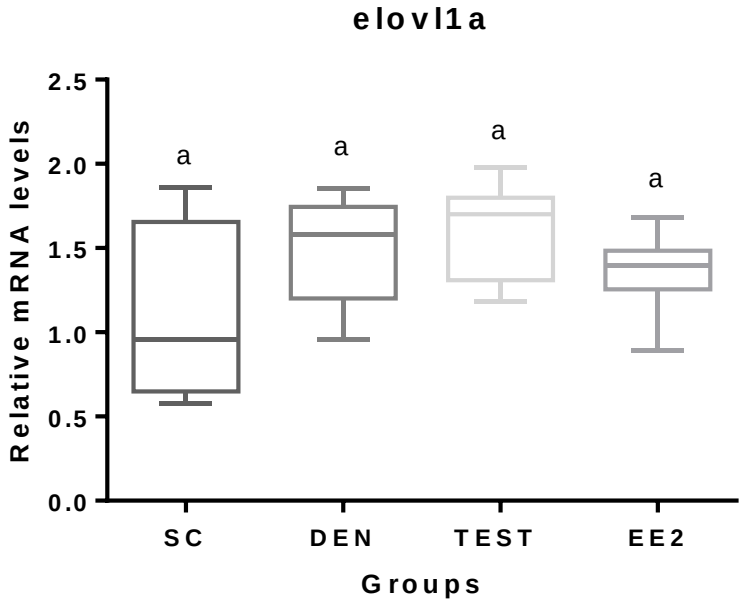


Figure 16. Relative mRNA levels of *elovl1a* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Tukey test, comparing the experimental conditions.

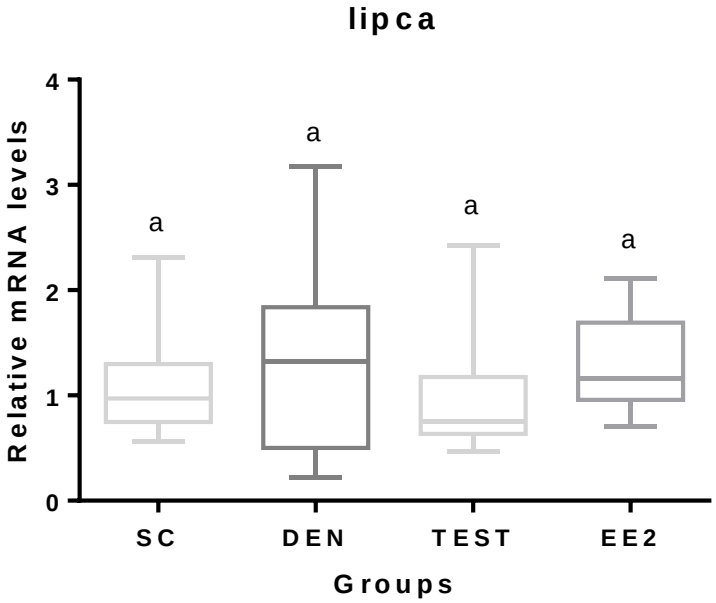


Figure 17. Relative mRNA levels of *lipca* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

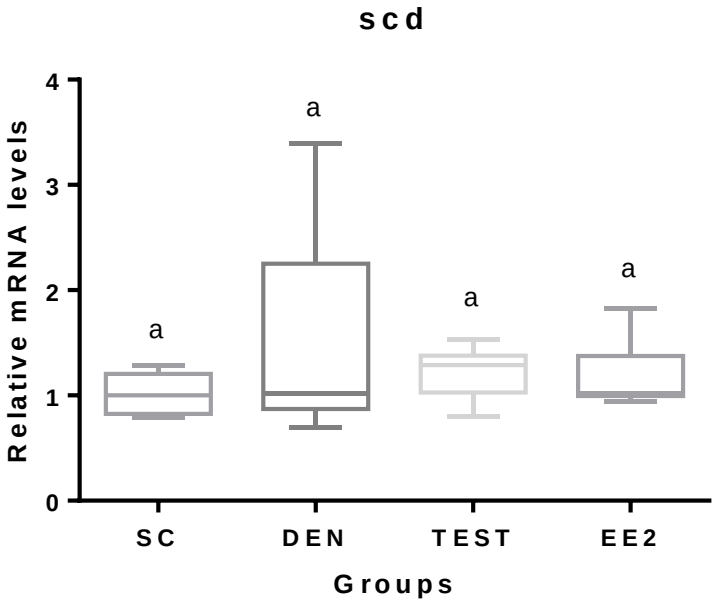


Figure 18. Relative mRNA levels of *scd* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

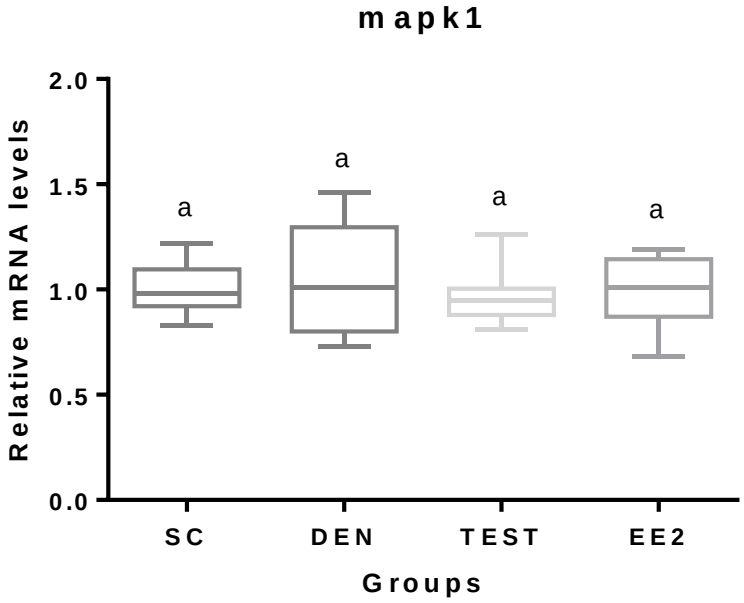


Figure 19. Relative mRNA levels of *mapk1* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

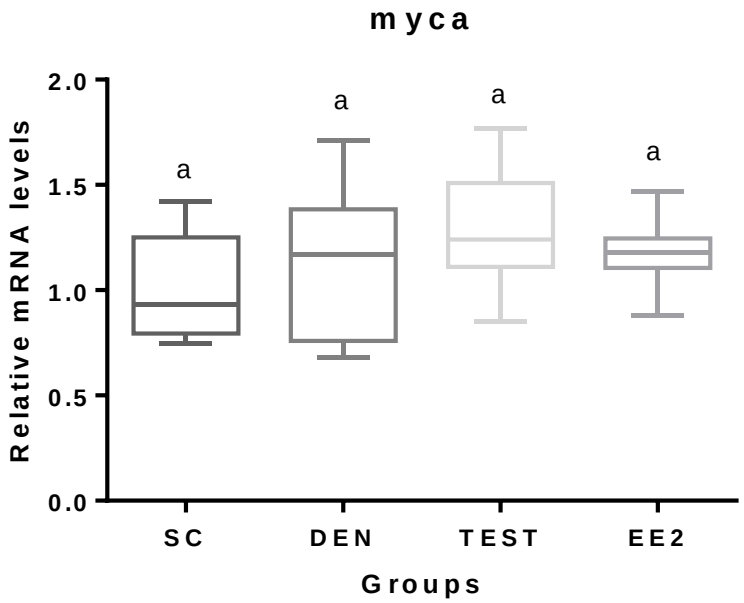


Figure 20. Relative mRNA levels of *myca* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

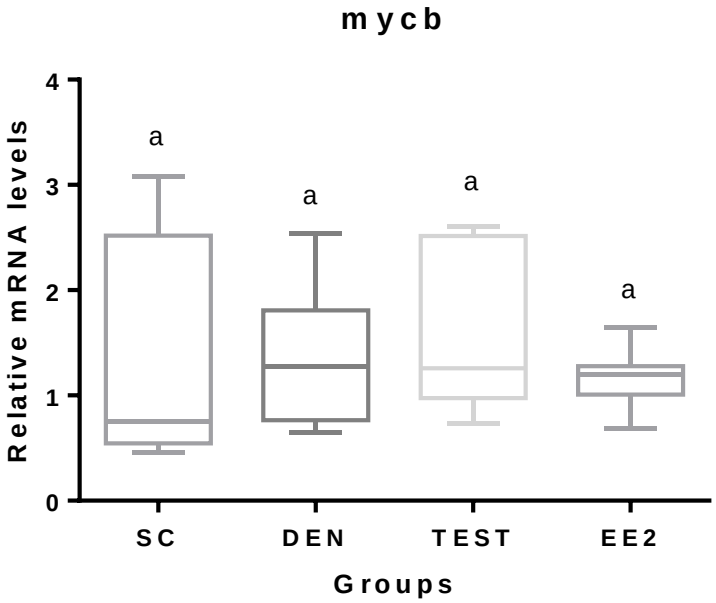


Figure 21. Relative mRNA levels of *mycb* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Mann-Whitney test with Bonferroni significance, comparing the experimental conditions.

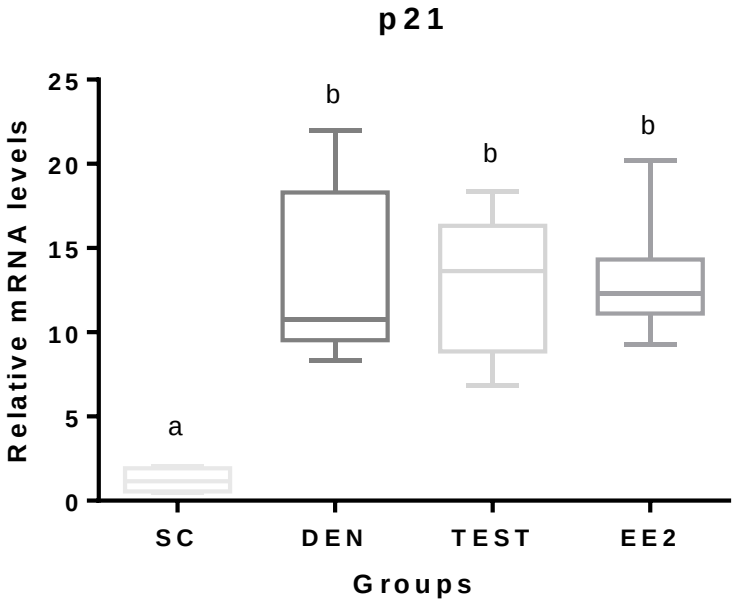


Figure 22. Relative mRNA levels of *p21* gene in Solvent Control (SC), Diethylnitrosamine (DEN), Diethylnitrosamine plus Testosterone (TEST), and Diethylnitrosamine plus Ethinylestradiol (EE2) groups. Significant differences (p-value <0.05) were calculated by one-way ANOVA, followed by Tukey test, comparing the experimental conditions.

4. Discussion

Here we start testing two fundamental hypotheses. One is that if zebrafish larvae are exposed to the initiating genotoxic carcinogen DEN, then disruptions occur in growth, liver development, and cell division and lipid metabolism molecular machinery (gene expression). The other one is that if there is a co-exposure to estrogenic or androgenic stimuli during the early stages of tumour initiation with DEN, then some of the effects of this carcinogen can be modulated (mitigated or exacerbated).

The fundamental rationale behind the two hypotheses are data from the literature supporting, on the one hand, a relation between tumour initiation and promotion in metabolic pathways, including energy/lipid-related, and, on the other hand, a role of androgenic and estrogenic steroids in carcinogenesis, namely in liver.

4.1. Fish Weight and Length and Liver Volume Estimation

The larval weight was not altered by the exposure to DEN or to DEN mixed with the androgen (TEST) or oestrogen (EE2), suggesting that irrespective of whatever mechanistic impacts that DEN may trigger in the larvae, during tumour initiation, they do not translate into growth disturbances (as evaluated by the animal mass), at this age and with this exposure period.

In what concerns biometric parameters related with length, there are differences to be reported. The results showed that all the lines that include point 5 (tail) have lower values in all the groups exposed to diethylnitrosamine (DEN, TEST and EE2) when compared to SC. This result support that the exposure to DEN leads to a short length of the tail, possibly inhibiting the development of this body part. To our best knowledge, only a recent study report impacts of the tumour initiating DEN in zebrafish length (Huang *et al*., 2018). In that study the authors stress the fact that seldom the developmental toxicity of DEN has been evaluated. Despite these data of Huang *et al.* (2018), they had a different design (larvae were exposed from 7 hpf and evaluated at 72 hpf), the authors reported a decreased length too.
In addition to the later study, we looked at other experimental contexts, Li *et al.* (2017a) also reported lower values on all the lines that include point 5 (as here) in zebrafish exposed to 4 mg/L of $CdCl_2$. In zebrafish exposed to different concentrations of pesticide glyphosate (a probable human carcinogen) there were

significant decreases in body length, regardless the concentration (Bridi *et al.*, 2017).

The underlying mechanisms of such type of impacts on length are unknown, and our data backs the pertinence to study them, namely in a carcinogenesis context, Huang *et al.* (2018) suggested some connection with excessive oxidative stress.
Here, we specifically studied the liver because it is a well-established main target for DEN genotoxicity (Shirakami *et al.*, 2012; Huang *et al.*, 2018). We must stress that the liver was already formed and growing at this stage of development (our assay ended with 5 dpf, i.e., 120 hpf larvae). This is in line with the reports that, as early as 32 hpf, the zebrafish liver primordium is expressing well-established markers of hepatocytes (Korzh *et al.*, 2001). Additionally, the establishment, growth and maturation of the biliary cells/tree concurs with hepatocyte differentiation, between 3 and 5 dpf (Wilkins & Pack, 2013). At 76 hpf liver (pancreas, and gut) are fully formed and attains in adult configuration at 96 hpf (Field *et al.*, 2003a; Field *et al.*, 2003b). Overall, data supports zebrafish liver is metabolic capable at the studied ages – including of expressing cytochrome P450 activity, especially after 72 hpf (Wilkins & Pack, 2013; Verbueken *et al.*, 2017) – which is important in the context of bio-activation of initiators, as happen with DEN. Our results showed that larvae exposed only to DEN had an increased volume of the liver, supporting that contact with the initiator either triggered hepatocellular hyperplasia and/or hypertrophy, what is confirmed by the qualitative analysis of histological images. Addressing which of underlying processes occurred needs another stereological approach, using other techniques. Determination of a proliferative hepatocytic index by immunohistochemistry against the proliferating cell nuclear antigen could be added to mechanistically address the current liver volume findings. Irrespective of the mechanism that was triggered by DEN, the parallel puzzling and biologically interesting finding here was that either the androgen or the oestrogen counteracted the DEN effect. The groups TEST and EE2 do not differ from the group DEN (and from the solvent only group too) in what regards the liver volume. An integration with literature findings is deserved.
In four-week-old mice, it was shown that DEN increases cell proliferation in the early stages of hepatocarcinogenesis (Arboatti *et al.*, 2018); to our knowledge this has not been well established yet in fish. Our data in zebrafish are perfectly in line with the DEN effects in mice, because hepatocellular proliferation may translate into increased liver mass, as seen in our study. Experimental studies, also in mice,

where either gonadectomy or hormonal injection was tested, strongly supported the hormone dependency of DEN induced liver tumours, with exposure (by injection) to 17β-estradiol decreasing incidence, multiplicity and size of liver tumours (Nakatani *et al.*, 2001). If such protective effect of oestrogens is also valid for zebrafish, this would explain why in our study ethinylestradiol neutralized the DEN effects.

In our assay, it is more cumbersome to interpret why the exposure to androgen also counteracted the baseline effect of DEN. For example, according to the cited study of Nakatani *et al.* (2001), liver tumours "positively" respond to testosterone. One elegant explanation could rely on the findings from studies with fish hepatocytes, which reveal that testosterone may elicit typical "estrogenic actions", via binding to oestrogen receptors (Lopes *et al.*, 2017). Additionally, it is known that testosterone is an aromatizable androgen that upregulates its own aromatization, namely in zebrafish (Mouriec *et al.*, 2009). In conclusion, a possible direct action of testosterone and/or its aromatization in oestradiol could be the explanatory basis of the neutralization of the DEN effect on liver volume in our TEST group. The idea gains further plausibility knowing that brain aromatase (*cyp19a1b*), is highly expressed during early development of zebrafish (Ulhaq & Kishida, 2018).

4.2. Quantification of mRNA

Regarding molecular analysis, two genes showed significant differences between experimental groups. So, overall, DEN did not impact on most selected genes.

The *acaa2* gene is greatly expressed in DEN group in comparison with SC group. Up-regulation of this gene in zebrafish has been found in other contexts. For example, after exposure to doxycycline, *acaa2* gene reported a higher expression in treatment group when compared to control group (Yao *et al.*, 2018).

This up-regulation means that the lipid metabolism suffered an alteration due to the presence of DEN. β-oxidation and lipolysis are being promoted, reducing fatty acids content, in order to provide the energy that tumour cells need to proliferate and survive (Peng *et al.*, 2018; Yang *et al.*, 2018). It is also seen that the hormones had an impact on this gene expression, because the groups exposed simultaneously to DEN and one of the hormones and no differences to SC group,

suggesting that, somehow, the presence of hormones reduced the effect of DEN and brought the two treatment groups (TEST and EE2) closer to the control group.

The high metabolic demands of cancer cells- and presumptively of initiated and transformed cells too - justify the importance that metabolic pathways assume in cancer cells survival, especially those pathways related to lipid metabolism (Pope *et al.*, 2019).
Abnormal expression of several key genes is commonly seen in many types of human tumours (Pope *et al.*, 2019). In general, HCC tumours present reduced rates of β-oxidation, but some cases of HCC are characterized by high rates of this process (Berndt *et al.*, 2019; Pope *et al.*, 2019).

The other gene that reported alterations is *p21*, and our results show that it is higher expressed in DEN and DEN + hormones groups than in SC group. This is consistent with the discoveries of Zhang *et al.* (2009) that reports up-regulated expression of *p21* in HCC tissues when compared to adjacent hepatic tissues, opposing to other articles that reported down-regulated expression in HCC cases (Soares *et al.*, 2012). Furthermore, it is possible to conclude that both hormones had no impact on the expression of this gene – contrarily to *acaa2* - because they had the same result as DEN when acts alone. If *p21* is highly expressed the route of cell division from G1 phase to S phase is delayed, so the cell can repair DNA damages before replication and propagation of genetic errors (Soares *et al.*, 2012).

Genes *mapk1, myca, mycb, elovl1a, lipca* and *scd* did not show significant differences. It would be expected that *mapk1, myc* genes, *lipca* and *scd* would be up-regulated in treatment groups. *myc* genes are altered , because several studies suggest that these genes are highly expressed in the presence of a chemically-induced hepatocarcinogenesis (Ito *et al.*, 1998; Mirbahai *et al.*, 2011; Nguyen *et al.*, 2011; Li *et al.*, 2013; Yao *et al.*, 2018). On the other hand, it was expected that *elovl1a* would be down-regulated in HCC (Yao *et al.*, 2018). Yet, our study deals with early stages of cell initiation and not with organ tumours. So, it is plausible to think that, initially, many of the gene impacts seen in progressing or in only well differentiated tumour did not emerged yet.

Our results suggest that, in this study, regarding lipid metabolism, only fatty acid β-oxidation pathway is increased and that there are no differences in lipid

mobilization and fatty acids synthesis. Regarding tumour pathways, the only difference resides in *p21*, that here exerts an oncogenic and an anti-apoptotic roles, since it is higher expressed on the groups that are exposed to the carcinogenic agent.

In sum, our results confirm that the exposure to chemical compounds like DEN affects the morphology of the exposed individuals, like decreased body length, has impacts on lipid and tumour pathways and that the presence of oestrogens and androgens can counteract the effects of DEN.

5. Conclusions

This study aimed to test two central hypotheses. First, understand if zebrafish larvae exposed to the carcinogenic agent DEN report impacts on growth, liver development and on lipid metabolism. The second one is if the co-exposure to DEN and oestrogen/androgen during early stages of development has impacts, negative or positive, on tumour initiation. These two hypotheses are based on the literature already available that suggests that there is a connection between tumour initiation and promotion of lipid-related pathways, and that androgens and oestrogens have an important role in hepatocarcinogenesis.

Biometric and morphological analyses demonstrated that the exposure to DEN for 48 h lead to a decrease in body's length, confirming that this compound can inhibit or slow the development of the individuals. Additionally, the exposure to DEN only resulted in an increased volume of the total liver, suggesting that the contact with the initiator triggered hepatocellular proliferation. Our results suggest that ethinylestradiol neutralized DEN effects. However, it is harder to understand and consequently to explain why the co-exposure to DEN and androgens had the same result as the co-exposure to DEN and oestrogens. This result can be explained by some studies, that report that testosterone may mimic oestrogens, by binding to oestrogen receptors.
The molecular analysis showed that, overall, DEN did not impact on most selected genes. However, it was possible to assume that the presence of a carcinogenic agent lead to the activation of a pathway involved in fatty acids β-oxidation, due to the energy demands of transformed cells. Our study is focused in early stages of cell initiation and does not deal with organ tumours. This means that, probably, the impacts of these genes are only seen in progressing level or in well differentiated tumours.

To conclude, our results confirm that the exposure to chemical compounds like DEN affects the morphology of the exposed individuals, like decreased body length, has impacts on lipid and tumour pathways and that the presence of oestrogens and androgens can counteract the effects of DEN.

There are few articles focusing on the link between HCC and lipid metabolism. Thus, there is a long way to go in this subject until better knowledge is reached. More genes, that are associated with other lipid and hepatocarcinogenesis pathways that were not addressed in this study, can be assessed. Also,

complementary techniques, such as immunohistochemistry, should be performed to determine proliferative hepatocytic index.

This theme is of outrageous importance because, as referred before in this dissertation, the incidence of liver cancers is increasing every year and HCC, especially, is responsible for several deaths every day worldwide. The continuous research using not only zebrafish, but also other models will, hopefully, lead to a deeper knowledge about the mechanisms behind HCC and, hopefully, be translated in a more efficient risk evaluation, prevention, detection and treatments.

6. References

Andersen, C., Jensen, J., & Ørntoft, T. 2004. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Research, 64*(15), 5245-5250.

Andrisani, O. M., Studach, L., & Merle, P. 2011. Gene Signatures in Hepatocellular Carcinoma (HCC). *Seminars in Cancer Biology, 21*(1), 4-9.

Arboatti, A. S., Lambertucci, F., Sedlmeier, M. G., Pisani, G., Monti, J., lvarez, M. d. L., Frances, D. E. A., Ronco, M. T., & Carnovale, C. E. 2018. Diethylnitrosamine Increases Proliferation in Early Stages of Hepatic Carcinogenesis in Insulin-Treated Type 1 Diabetic Mice. *BioMed Research International, 2018*, 1-10.

Arvanitis, C., & Felsher, D. W. 2006. Conditional Transgenic Models Define How MYC Initiates and Maintains Tumorigenesis. *Seminars in Cancer Biology, 16*(4), 313-317.

Barone, M., Margiotta, M., Scavo, M. P., Gentile, A., Francioso, D., Papagni, S., Castellaneta, A., Mallamaci, R., Di Leo, A., & Francavilla, A. 2009. Possible Involvement of Androgen Receptor Alterations in Hepatocarcinogenesis. *Digestive and Liver Disease, 41*(9), 665-670.

Baumann, P. C., & Okihiro, M. S. 2000. Cancer. In G. K. Ostrander (Ed.), *The Laboratory Fish* (1st ed., 591-616): Academic Press.

Bechmann, L. P., Hannivoort, R. A., Gerken, G., Hotamisligil, G. S., Trauner, M., & Canbay, A. 2012. The Interaction of Hepatic Lipid and Glucose Metabolism in Liver Diseases. *Journal of Hepatology, 56*(4), 952-964.

Beloribi-Djefaflia, S., Vasseur, S., & Guillaumond, F. 2016. Lipid Metabolic Reprogramming in Cancer Cells. *Oncogenesis, 5*(1), 1-10.

Berndt, N., Eckstein, J., Heucke, N., Gajowski, R., Stockmann, M., Meierhofer, D., & Holzütter, H.-G. 2019. Characterization of Lipid and Lipid Droplet Metabolism in Human HCC. *Cells, 8*(5), 512-530.

Blanc, M. 2016. Time-Dependent Toxicity of PCB 126, PFOS and PFHxA Alone and in Mixture on Zebrafish Embryos (*Danio rerio*) on Key-Pathways of Metabolism. (MSc). Orebro University, Sweden.

Bopp, K., Lettieri, T., & Minuzzo, M. 2007. The Zebrafish (*Danio rerio*) an Emerging Model Organism in the Environmental Field. EU

Boroughs, L. K., & DeBerardinis, R. J. 2015. Metabolic Pathways Promoting Cancer Cell Survival and Growth. *Nature Cell Biology, 17*(4), 351-359.

Bridi, D., Altenhofen, S., Gonzalez, J. B., Reolon, G. K., & Bonan, C. D. 2017. Glyphosate and Roundup® Alter Morphology and Behavior in Zebrafish. *Toxicology, 392*, 32-39.

Bunton, T. E. 1990. Hepatopathology of Diethylnitrosamine in the Medaka (*Oryzias latipes)* Following Short-Term Exposure. *Toxicology Pathology, 18*(2), 313-323.

Calcagno, D.-Q., Leal, M.-F., Assumpcao, P.-P., Smith, M.-A.-C., & Burbano, R.-R. 2008. MYC and Gastric Adenocarcinoma Carcinogenesis. *World Journal of Gastroenterology, 14*(39), 5962-5968.

Chaturantabut, S., Shwartz, A., Evason, K. J., Cox, A. G., Labella, K., Schepers, A. G., Yang, S., Acuña, M., Houvras, Y., Mancio-Silva, L., Romano, S., Gorelick, D. A., Cohen, D. E., Zon, L. I., Bhatia, S. N., North, T. E., & Goessling, W. 2019. Estrogen Activation of G-Protein-Coupled Estrogen Receptor 1 Regulates Phosphoinositide 3-Kinase and mTOR Signaling to Promote Liver Growth in Zebrafish and Proliferation of Human hepatocytes. *Gastroenterology, 156*(6), 1788-1804.

Chen, Y., & Olopade, O. I. 2008. MYC in Breast Tumor Progression. *Expert Review of Anticancer Therapy, 8*(10), 1689-1698.

Chen, Y. Y., Zhu, J. Y., & Chan, K. M. 2014. Effects of Cadmium on Cell Proliferation, Apoptosis, and Proto-Oncogene Expression in Zebrafish Liver Cells. *Aquatic Toxicology, 157*, 196-206.

Cheng, C., Geng, F., Cheng, X., & Guo, D. 2018. Lipid Metabolism Reprogramming and Its Potential Targets in Cancer. *Cancer Communications, 38*(1), 27.

Cooper, G. M. 2000. The Development and Causes of Cancer. In Sunderland (Ed.), *The Cell: A Molecular Approach* (4th ed.): ASM Press.

Corral, M., Paris, B., Guguen-Guillouzo, C., Corcos, D., Kruh, J., & Defer, N. 1988. Increased Expression of the N-myc Gene During Normal and Neoplastic Rat Liver Growth. *Experimental Cell Research, 174*(1), 107-115.

Costa Raiol, L. C., Figueira Silva, E. C., Mendes da Fonseca, D., Leal, M. F., Guimaraes, A. C., Calcagno, D. Q., Khayat, A. S., Assumpcao, P. P., de Arruda Cardoso Smith, M., & Burbano, R. R. 2008. Interrelationship between MYC Gene Numerical Aberrations and Protein Expression in Individuals from Northern Brazil with Early Gastric Adenocarcinoma. *Cancer Genetics and Cytogenetics, 181*(1), 31-35.

Currie, E., Schulze, A., Zechner, R., Walther, T. C., & Farese, R. V., Jr. 2013. Cellular Fatty Acid Metabolism and Cancer. *Cell Metabolism, 18*(2), 153-161.

Dalvai, M., & Bystricky, K. 2010. Cell Cycle and Anti-Estrogen Effects Synergize to Regulate Cell Proliferation and ER Target Gene Expression. *PLoS One, 5*(6), 1-9.

De Maria, N., Manno, M., & Villa, E. 2002. Sex Hormones and Liver Cancer. *Molecular and Cellular Endocrinology, 193*(1-2), 59-63.

De Matteis, S., Ragusa, A., Marisi, G., De Domenico, S., Casadei Gardini, A., Bonaf, M., & Giudetti, A. M. 2018. Aberrant Metabolism in Hepatocellular Carcinoma Provides Diagnostic and Therapeutic Opportunities. *Oxidative Medicine and Cellular Longevity, 2018*, 13.

den Hertog, J. 2016. Tumor Suppressors in Zebrafish: From TP53 to PTEN and Beyond. *Advances in Experimental Medicine and Biology, 916*, 87-101.

Dhanasiri, A. K. S., Fernandes, J. M. O., & Kiron, V. 2013. Liver Transcriptome Changes in Zebrafish During Acclimation to Transport-Associated Stress. *PLoS One, 8*(6), 1-12.

Di Maio, M., De Maio, E., Morabito, A., D'Aniello, R., De Feo, G., Gallo, C., & Perrone, F. 2006. Hormonal Treatment of Human Hepatocellular Carcinoma. *Annals of the New York Academy of Sciences, 1089*, 252-261.

Dubińska-Magiera, M., Daczewska, M., Lewicka, A., Migocka-Patrzałek, M., Niedbalska-Tarnowska, J., & Jagla, K. 2016. Zebrafish: A Model for the Study of Toxicants Affecting Muscle Development and Function. *International Journal of Molecular Sciences, 17*(11), 1-22.

El-Serag, H. B., & Rudolph, K. L. 2007. Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis. *Gastroenterology, 132*(7), 2557-2576.

Elba, S., Buongiorno, G. P., Caruso, M. L., Noviello, M. R., & Manghisi, O. G. 2002. Main Characteristics of Hepatocellular Carcinoma and Cirrhosis and Therapeutic Approaches. *Current Pharmaceutical Design, 8*(11), 1007-1011.

Etchin, J., Kanki, J., & Look, A. 2011. Zebrafish as a Model for the Study of Human Cancer. *Methods in cell biology, 105*, 309-337.

Ferretti, G., Felici, A., & Cognetti, F. 2007. The Protective Side of Progesterone. *Breast Cancer Research, 9*(6), 402.

Fetter, E., Smetanova, S., Baldauf, L., Lidzba, A., Altenburger, R., Schuttler, A., & Scholz, S. 2015. Identification and Characterization of Androgen-Responsive Genes in Zebrafish Embryos. *Environmental Science & Technology, 49*(19), 11789-11798.

Field, H. A., Dong, P. D., Beis, D., & Stainier, D. Y. 2003a. Formation of the Digestive System in Zebrafish. II. Pancreas Morphogenesis. *Developmental Biology, 261*(1), 197-208.

Field, H. A., Ober, E. A., Roeser, T., & Stainier, D. Y. 2003b. Formation of the Digestive System in Zebrafish. I. Liver Morphogenesis. *Developmental Biology, 253*(2), 279-290.

Ghouri, Y.A., Mian, I., Rowe, J.H. 2017. Review of Hepatocellular Carcinoma: Epidemiology, Etiology and Carcinogenesis. *Journal of Carcinogenesis*, *16.*

Gundersen, H. J., & Jensen, E. B. 1987. The Efficiency of Systematic Sampling in Stereology and Its Prediction. *Journal of Microscopy, 147*(3), 229-263.

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica, 4*(1), 1-9.

Hanahan, D., & Weinberg, R. A. 2000. The Hallmarks of Cancer. *Cell, 100*(1), 57-70.

Hinton, D. E., Lauren, D. J., Teh, S. J., & Giam, C. S. 1988. Cellular Composition and Ultrastructure of Hepatic Neoplasms Induced by Diethylnitrosamine in *Oryzias latipes*. *Marine Environmental Research, 24*(1), 307-310.

Hoffmann, K., Shibo, L., Xiao, Z., Longerich, T., Buchler, M. W., & Schemmer, P. 2011. Correlation of Gene Expression of ATP-Binding Cassette Protein and Tyrosine Kinase Signaling Pathway in Patients with Hepatocellular Carcinoma. *Anticancer Research, 31*(11), 3883-3890.

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. J., White, S., Chow, W., Kilian, B., Quintais, L. T., Guerra-Assuncao, J. A., Zhou, Y., Gu, Y., Yen, J., Vogel, J. H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S. F., Laird, G. K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer,

L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C. M., Ersan-Urun, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberlander, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S. C., Carter, N. P., Harrow, J., Ning, Z., Herrero, J., Searle, S. M., Enright, A., Geisler, R., Plasterk, R. H., Lee, C., Westerfield, M., de Jong, P. J., Zon, L. I., Postlethwait, J. H., Nusslein-Volhard, C., Hubbard, T. J., Roest Crollius, H., Rogers, J., & Stemple, D. L. 2013. The Zebrafish Reference Genome Sequence and Its Relationship to the Human Genome. *Nature, 496*(7446), 498-503.

Hua, H., Zhang, H., Kong, Q., & Jiang, Y. 2018. Mechanisms for Estrogen Receptor Expression in Human Cancer. *Experimental Hematology & Oncology, 7*(1), 24-35.

Huang, D., Li, H., He, Q., Yuan, W., Chen, Z., & Yang, H. 2018. Developmental Toxicity of Diethylnitrosamine in Zebrafish Embryos/Juveniles Related to Excessive Oxidative Stress. *Water, Air, and Soil Pollution, 229*(3), 81-81.

Huang, X., Zhou, L., & Gong, Z. 2012. Liver Tumor Models in Transgenic Zebrafish: An Alternative in Vivo Approach to Study Hepatocarcinogenes. *Future Oncology, 8*(1), 21-28.

Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., & Hori, M. 1998. Activation of Mitogen-Activated Protein Kinases/Extracellular Signal-Regulated Kinases in Human Hepatocellular Carcinoma. *Hepatology, 27*(4), 951-958.

Kalra, M., Mayes, J., Assefa, S., Kaul, A. K., & Kaul, R. 2008. Role of Sex Steroid Receptors in Pathobiology of Hepatocellular Carcinoma. *World Journal of Gastroenterology, 14*(39), 5945-5961.

Kaposi-Novak, P., Libbrecht, L., Woo, H. G., Lee, Y. H., Sears, N. C., Coulouarn, C., Conner, E. A., Factor, V. M., Roskams, T., & Thorgeirsson, S. S. 2009. Central

Role of c-MYC During Malignant Conversion in Human Hepatocarcinogenesis. *Cancer research, 69*(7), 2775-2782.

Khalaf, A. M., Fuentes, D., Morshid, A. I., Burke, M. R., Kaseb, A. O., Hassan, M., Hazle, J. D., & Elsayes, K. M. 2018. Role of Wnt/B-Catenin Signaling in Hepatocellular Carcinoma, Pathogenesis, and Clinical Significance. *Journal of Hepatocellular Carcinoma, 5*, 61-73.

Kim, C. M., Koike, K., Saito, I., Miyamura, T., & Jay, G. 1991. HBx Gene of Hepatitis B Virus Induces Liver Cancer in Transgenic Mice. *Nature, 351*(6324), 317-320.

Korzh, S., Emelyanov, A., & Korzh, V. 2001. Developmental Analysis of Ceruloplasmin Gene and Liver Formation in Zebrafish. *Mechanisms of Development, 103*(1-2), 137-139.

Lam, S. H., & Gong, Z. 2006. Modeling Liver Cancer Using Zebrafish: A Comparative Oncogenomics Approach. *Cell Cycle, 5*(6), 573-577.

Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., & Braunbeck, T. 2009. Is the Fish Embryo Toxicity Test (FET) with the Zebrafish (*Danio rerio*) a Potential Alternative for the Fish Acute Toxicity Test? *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology, 149*(2), 196-209.

Lee, L. L., Lee, J. S. C., Waldman, S., Casper, R. F., & Grynpas, M. 2002. Polycyclic Aromatic Hydrocarbons Present in Cigarette Smoke Cause Bone Loss in an Ovariectomized Rat Model. *Bone, 30*, 917-923.

Lemaigre, F., & Zaret, K. S. 2004. Liver Development Update: New Embryo Models, Cell Lineage Control, and Morphogenesis. *Current Opinion in Genetics & Development, 14*(5), 582-590.

Letrado, P., de Miguel, I., Lamberto, I., Díez-Martínez, R., & Oyarzabal, J. 2018. Zebrafish: Speeding up the Cancer Drug Discovery Process. *Cancer Research, 78*(21), 6048-6058.

Li, G., Ye, H., Su, G., Han, Z., Xie, C., Zhou, B., Letcher, R. J., Giesy, J. P., Yu, H., & Liu, C. 2017a. Establishment of a Three-Step Method to Evaluate Effects of Chemicals on Development of Zebrafish Embryo/Larvae. *Chemosphere, 186*, 209-217.

Li, Y., Li, H., Spitsbergen, J. M., & Gong, Z. 2017b. Males Develop Faster and More Severe Hepatocellular Carcinoma Than Females in Krasv12 Transgenic Zebrafish. *Scientific Reports, 7*, 1-10.

Li, Z., Zheng, W., Wang, Z., Zeng, Z., Zhan, H., Li, C., Zhou, L., Yan, C., Spitsbergen, J. M., & Gong, Z. 2013. A Transgenic Zebrafish Liver Tumor Model with Inducible MYC Expression Reveals Conserved MYC Signatures with Mammalian Liver Tumors. *Disease Models & Mechanisms, 6*(2), 414-423.

Liu, Y., Yin, T., Feng, Y., Cona, M. M., Huang, G., Liu, J., Song, S., Jiang, Y., Xia, Q., Swinnen, J. V., Bormans, G., Himmelreich, U., Oyen, R., & Ni, Y. 2015. Mammalian Models of Chemically Induced Primary Malignancies Exploitable for Imaging-Based Preclinical Theragnostic Research. *Quantitative Imaging in Medicine and Surgery, 5*(5), 708-729.

Llovet, J. M., Burroughs, A., & Bruix, J. 2003. Hepatocellular Carcinoma. *Lancet, 362*(9399), 1907-1917.

Lodish, H., Berk, A., & Zipursky, S. L. 2000. Proto-Oncogenes and Tumor-Suppressor Genes. In *Molecular Cell Biology* (4th ed.): W. H. Freeman.

Long, J., Zhang, C.-J., Zhu, N., Du, K., Yin, Y.-F., Tan, X., Liao, D.-F., & Qin, L. 2018. Lipid Metabolism and Carcinogenesis, Cancer Development. *American Journal of Cancer Research, 8*(5), 778-791.

Lopes, C., Malhao, F., Guimaraes, C., Pinheiro, I., Goncalves, J. F., Castro, L. F. C., Rocha, E., & Madureira, T. V. 2017. Testosterone-Induced Modulation of Peroxisomal Morphology and Peroxisome-Related Gene Expression in Brown Trout (*Salmo trutta fario*) Primary Hepatocytes. *Aquatic Toxicology, 193*, 30-39.

Lu, G. D., & Hooi, S. C. 2017. Lipid Metabolism in Liver Cancer In *Updates in Liver Cancer*: IntechOpen.

Lu, J. W., Hsia, Y., Tu, H. C., Hsiao, Y. C., Yang, W. Y., Wang, H. D., & Yuh, C. H. 2011. Liver Development and Cancer Formation in Zebrafish. *Birth Defects Research Part C - Embryo Today, 93*(2), 157-172.

Madureira, T. V., Cruzeiro, C., Rocha, M. J., & Rocha, E. 2011. The Toxicity Potential of Pharmaceuticals Found in the Douro River Estuary (Portugal)--Experimental Assessment Using a Zebrafish Embryo Test. *Environmental Toxicology and Pharmacology, 32*(2), 212-217.

Malarkey, D. E., Johnson, K., Ryan, L., Boorman, G., & Maronpot, R. R. 2005. New Insights into Functional Aspects of Liver Morphology. *Toxicologic Pathology, 33*(1), 27-34.

Mirbahai, L., Williams, T. D., Zhan, H., Gong, Z., & Chipman, J. K. 2011. Comprehensive Profiling of Zebrafish Hepatic Proximal Promoter CpG Island

Methylation and Its Modification During Chemical Carcinogenesis. *BMC Genomics, 12*(3), 3-19.

Montella, M., Crispo, A., & Giudice, A. 2011. HCC, Diet and Metabolic Factors: Diet and HCC. *Hepatitis Monthly, 11*(3), 159-162.

Montella, M., D'Arena, G., Crispo, A., Capunzo, M., Nocerino, F., Grimaldi, M., Barbieri, A., D'Ursi, A. M., Tecce, M. F., Amore, A., Galdiero, M., Ciliberto, G., & Giudice, A. 2015. Role of Sex Hormones in the Development and Progression of Hepatitis B Virus-Associated Hepatocellular Carcinoma. *International Journal of Endocrinology, 2015*, 1-9.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., & Koike, K. 1998. The Core Protein of Hepatitis C Virus Induces Hepatocellular Carcinoma in Transgenic Mice. *Nature Medicine, 4*(9), 1065-1067.

Mouriec, K., Gueguen, M.-M., Manuel, C., Percevault, F., Thieulant, M.-L., Pakdel, F., & Kah, O. 2009. Androgens Upregulate *cyp19a1b* (Aromatase B) Gene Expression in the Brain of Zebrafish (*Danio rerio*) through Estrogen Receptors1. *Biology of Reproduction, 80*(5), 889-896.

Nakagawa, H., Hayata, Y., Kawamura, S., Yamada, T., Fujiwara, N., & Koike, K. 2018. Lipid Metabolic Reprogramming in Hepatocellular Carcinoma. *Cancers, 10*(11), 447-461.

Nakatani, T., Roy, G., Fujimoto, N., Asahara, T., & Ito, A. 2001. Sex Hormone Dependency of Diethylnitrosamine-Induced Liver Tumors in Mice and Chemoprevention by Leuprorelin. *Japanese Journal of Cancer Research, 92*(3), 249-256.

Nakra, B.R., Lee, F.E., Gaind, R. 1973. Risk of Paracetamol Abuse. *Lancet, 2 (7826)*, 451-452.

Newell, P., Villanueva, A., Friedman, S. L., Koike, K., & Llovet, J. M. 2008. Experimental Models of Hepatocellular Carcinoma. *Journal of Hepatology, 48*(5), 858-879.

Nguyen, A. T., Emelyanov, A., Koh, C. H. V., Spitsbergen, J. M., Lam, S. H., Mathavan, S., Parinov, S., & Gong, Z. 2011. A High Level of Liver-Specific Expression of Oncogenic Kras(V12) Drives Robust Liver Tumorigenesis in Transgenic Zebrafish. *Disease Models & Mechanisms, 4*(6), 801-813.

Ohkoshi, S., Yano, M., & Matsuda, Y. 2015. Oncogenic Role of P21 in Hepatocarcinogenesis Suggests a New Treatment Strategy. *World Journal of Gastroenterology, 21*(42), 12150-12156.

Ohnishi, S., Murakami, T., Moriyama, T., Mitamura, K., & Imawari, M. 1986. Androgen and Estrogen Receptors in Hepatocellular Carcinoma and in the Surrounding Noncancerous Liver Tissue. *Hepatology, 6*(3), 440-443.

Parkin, D. M., Bray, F., Ferlay, J., & Pisani, P. 2001. Estimating the World Cancer Burden: Globocan 2000. *International Journal of Cancer, 94*(2), 153-156.

Pelengaris, S., & Khan, M. 2003. The c-MYC Oncoprotein as a Treatment Target in Cancer and Other Disorders of Cell Growth. *Expert Opinion on Therapeutic Targets, 7*(5), 623-642.

Peng, M., Li, S., He, Q., Zhao, J., Li, L., & Ma, H. 2018. Proteomics Reveals Changes in Hepatic Proteins During Chicken Embryonic Development: An Alternative Model to Study Human Obesity. *BMC Genomics, 19-34*(1), 29-29.

Pfaffl, M. W. 2001. A New Mathematical Model for Relative Quantification in Real-Time RT-PCR. *Nucleic Acids Research, 29*(9).

Piccinin, E., Villani, G., & Moschetta, A. 2019. Metabolic Aspects in NAFLD, NASH and Hepatocellular Carcinoma: The Role of PGC1 Coactivators. *Nature Reviews Gastroenterology & Hepatology, 16*(3), 160-174.

Pope, E. D., 3rd, Kimbrough, E. O., Vemireddy, L. P., Surapaneni, P. K., Copland, J. A., 3rd, & Mody, K. 2019. Aberrant Lipid Metabolism as a Therapeutic Target in Liver Cancer. *Expert Opinion on Therapeutic Targets, 23*(6), 473-483.

Ravi, V., & Venkatesh, B. 2018. The Divergent Genomes of Teleosts. *Annual Review of Animal Biosciences, 6*(1), 47-68.

Rice, S., & Whitehead, S. A. 2006. Phytoestrogens and Breast Cancer-Promoters or Protectors? *Endocrine-Related Cancer, 13*(4), 995-1015.

Sant, K. E., & Timme-Laragy, A. R. 2018. Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo. *Current Environmental Health Reports, 5*(1), 125-133.

Santhakumar, K., Judson, E. C., Elks, P. M., McKee, S., Elworthy, S., van Rooijen, E., Walmsley, S. S., Renshaw, S. A., Cross, S. S., & van Eeden, F. J. 2012. A Zebrafish Model to Study and Therapeutically Manipulate Hypoxia Signaling in Tumorigenesis. *Cancer Research, 72*(16), 4017-4027.

Santos, N. P., Colaco, A. A., & Oliveira, P. A. 2017. Animal Models as a Tool in Hepatocellular Carcinoma Research: A Review. *Tumour Biology, 39*(3), 1-20.

Sarwar, R., Pierce, N., & Koppe, S. 2018. Obesity and Nonalcoholic Fatty Liver Disease: Current Perspectives. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 11*, 533-542.

Shang, S., Hua, F., & Hu, Z.-W. 2017. The Regulation of B-Catenin Activity and Function in Cancer: Therapeutic Opportunities. *Oncotarget, 8*(20), 33972-33989.

Shimizu, I., Yasuda, M., Mizobuchi, Y., Ma, Y.-R., Liu, F., Shiba, M., Horie, T., & Ito, S. 1998. Suppressive Effect of Oestradiol on Chemical Hepatocarcinogenesis in Rats. *Gut, 42*(1), 112-119.

Shirakami, Y., Gottesman, M. E., & Blaner, W. S. 2012. Diethylnitrosamine-Induced Hepatocarcinogenesis Is Suppressed in Lecithin:Retinol Acyltransferase-Deficient Mice Primarily through Retinoid Actions Immediately after Carcinogen Administration. *Carcinogenesis, 33*(2), 268-274.

Shive, H. R. 2013. Zebrafish Models for Human Cancer. *Veterinary Pathology, 50*(3), 468-482.

Si-Tayeb, K., Lemaigre, F. P., & Duncan, S. A. 2010. Organogenesis and Development of the Liver. *Developmental Cell, 18*(2), 175-189.

Soares, J., Castro, L. F., Reis-Henriques, M. A., Monteiro, N. M., & Santos, M. M. 2012. Zebrafish (*Danio rerio*) Life-Cycle Exposure to Chronic Low Doses of Ethinylestradiol Modulates p53 Gene Transcription within the Gonads, but Not Ner Pathways. *Ecotoxicology, 21*(5), 1513-1522.

Soni, N. K., Ross, A. B., Scheers, N., Savolainen, O. I., Nookaew, I., Gabrielsson, B. G., & Sandberg, A.-S. 2016. Eicosapentaenoic and Docosahexaenoic Acid-Enriched High Fat Diet Delays Skeletal Muscle Degradation in Mice. *Nutrients, 8*(9), 543-560.

Spitsbergen, J. M., Tsai, H. W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J. D., & Bailey, G. S. 2000. Neoplasia in Zebrafish (*Danio rerio*) Treated with N-Methyl-N'-Nitro-N-Nitrosoguanidine by Three Exposure Routes at Different Developmental Stages. *Toxicologic Pathology, 28*(5), 716-725.

Sukocheva, O. A. 2018. Estrogen, Estrogen Receptors, and Hepatocellular Carcinoma: Are We There Yet? *World Journal of Gastroenterology, 24*(1), 1-4.

Takahashi, Y., Kawate, S., Watanabe, M., Fukushima, J., Mori, S., & Fukusato, T. 2007. Amplification of c-myc and Cyclin D1 Genes in Primary and Metastatic Carcinomas of the Liver. *Pathology International, 57*(7), 437-442.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. 2012. Heavy Metal Toxicity and the Environment. *Experientia Supplementum (2012), 101*, 133-164.

Thorgeirsson, S. S., & Grisham, J. W. 2002. Molecular Pathogenesis of Human Hepatocellular Carcinoma. *Nature Genetics, 31*(4), 339-346.

Tu, T. Y., Hong, C. Y., Sasado, T., Kashiwada, S., & Chen, P. J. 2016. Early Life Exposure to a Rodent Carcinogen Propiconazole Fungicide Induces Oxidative Stress and Hepatocarcinogenesis in Medaka Fish. *Aquatic Toxicology, 170*, 52-61.

Ulhaq, Z. S., & Kishida, M. 2018. Brain Aromatase Modulates Serotonergic Neuron by Regulating Serotonin Levels in Zebrafish Embryos and Larvae. *Frontiers in Endocrinology, 9*.

Van Beneden, R. J., Henderson, K. W., Blair, D. G., Papas, T. S., & Gardner, H. S. 1990. Oncogenes in Hematopoietic and Hepatic Fish Neoplasms. *Cancer Research, 50*(17), 5671-5674.

van der Laan, J. W., Buitenhuis, W. H. W., Wagenaar, L., Soffers, A. E. M. F., van Someren, E. P., Krul, C. A. M., & Woutersen, R. A. 2016. Prediction of the Carcinogenic Potential of Human Pharmaceuticals Using Repeated Dose Toxicity Data and Their Pharmacological Properties. *Frontiers in Medicine, 3*(45).

Vanhauwaert, S., Van Peer, G., Rihani, A., Janssens, E., Rondou, P., Lefever, S., De Paepe, A., Coucke, P. J., Speleman, F., Vandesompele, J., & Willaert, A. 2014. Expressed Repeat Elements Improve RT-qPCR Normalization across a Wide Range of Zebrafish Gene Expression Studies. *PLoS One, 9*(10), 1-10.

Verbueken, E., Alsop, D., Saad, M. A., Pype, C., Van Peer, E. M., Casteleyn, C. R., Van Ginneken, C. J., Wilson, J., & Van Cruchten, S. J. 2017. In Vitro Biotransformation of Two Human CYP3A Probe Substrates and Their Inhibition During Early Zebrafish Development. *International Journal of Molecular Sciences, 18*(1), 217-233.

Vesselinovitch, S. D., Itze, L., Mihailovich, N., & Rao, K. V. 1980. Modifying Role of Partial Hepatectomy and Gonadectomy in Ethylnitrosourea-Induced Hepatocarcinogenesis. *Cancer Research, 40*(5), 1538-1542.

Wang, M., Han, J., Xing, H., Zhang, H., Li, Z., Liang, L., Li, C., Dai, S., Wu, M., Shen, F., & Yang, T. 2016. Dysregulated Fatty Acid Metabolism in Hepatocellular Carcinoma. *Hepatic Oncology, 3*(4), 241-251.

Wilkins, B. J., & Pack, M. 2013. Zebrafish Models of Human Liver Development and Disease. *Comprehensive Physiology, 3*(3), 1213-1230.

Willenbring, H., Sharma, A. D., Vogel, A., Lee, A. Y., Rothfuss, A., Wang, Z., Finegold, M., & Grompe, M. 2008. Loss of p21 Permits Carcinogenesis from

Chronically Damaged Liver and Kidney Epithelial Cells Despite Unchecked Apoptosis. *Cancer Cell, 14*(1), 59-67.

Yager, J. D., Jr., & Yager, R. 1980. Oral Contraceptive Steroids as Promoters of Hepatocarcinogenesis in Female Sprague-Dawley Rats. *Cancer Research, 40*(10), 3680-3685.

Yang, S., & Liu, G. 2017. Targeting the Ras/Raf/MEK/ERK Pathway in Hepatocellular Carcinoma. *Oncology Letters, 13*(3), 1041-1047.

Yang, Y., Fang, X., Yang, R., Yu, H., Jiang, P., Sun, B., & Zhao, Z. 2018. MiR-152 Regulates Apoptosis and Triglyceride Production in MECS Via Targeting ACAA2 and HSD17B12 Genes. *Scientific Reports, 8*(1), 1-10.

Yao, Y., Sun, S., Wang, J., Fei, F., Dong, Z., Ke, A. W., He, R., Wang, L., Zhang, L., Ji, M. B., Li, Q., Yu, M., Shi, G. M., Fan, J., Gong, Z., & Wang, X. 2018. Canonical Wnt Signaling Remodels Lipid Metabolism in Zebrafish Hepatocytes Following Ras Oncogenic Insult. *Cancer Research, 78*(19), 5548-5560.

Yin, A., Korzh, S., Winata, C. L., Korzh, V., & Gong, Z. 2011. Wnt Signaling Is Required for Early Development of Zebrafish Swimbladder. *PLoS One, 6*(3).

Yin, F., Sharen, G., Yuan, F., Peng, Y., Chen, R., Zhou, X., Wei, H., Li, B., Jing, W., & Zhao, J. 2017. TIP30 Regulates Lipid Metabolism in Hepatocellular Carcinoma by Regulating SREBP1 through the Akt/mTOR Signaling Pathway. *Oncogenesis, 6*(6), 1-9.

Zhang, M.-F., Zhang, Z.-Y., Fu, J., Yang, Y.-F., & Yun, J.-P. 2009. Correlation between Expression of p53, p21/WAF1, and MDM2 Proteins and Their Prognostic Significance in Primary Hepatocellular Carcinoma. *Journal of Translational Medicine, 7*(1), 110-118.

Zorn, A. M. 2008. Liver Development. In *Stembook* (1-26). Cambridge (MA).

7. Appendix

Appendix I

Histological processing to paraffin

One hour in each step:

1. Ethanol 70% (Proclínica, Portugal)
2. Ethanol 95% (Fábrica do Álcool, Portugal)
3. Ethanol 95% (Fábrica do Álcool, Portugal)
4. Ethanol 100% (Proclínica, Portugal)
5. Ethanol 100% (Proclínica, Portugal)
6. Ethanol 100% (Proclínica, Portugal)
7. Ethanol & Xylene (1:1)
8. Xylene (VWR, France)
9. Xylene (VWR, France)
10. Paraffin (Thermo Scientific, USA)
11. Paraffin (Thermo Scientific, USA)

Appendix II

Haematoxylin & Eosin Staining

1. Deparaffinize with xylene (10 minutes 2x)
2. Hydrate in ethanol – 100%, 95%, 70% (Proclínica, Portugal; Fábrica do Álcool, Portugal; Proclínica, Portugal) (5 minutes each)
3. Wash carefully in tap water (5 minutes)
4. Stain with Mayer Haematoxylin (Merck, Germany) (2 minutes)
5. Wash carefully in tap water (5 minutes)
6. Stain with aqueous 1% eosin (Bio-Optica, Italy) (2-5 minutes)
7. Wash quickly and carefully in tap water.
8. Dehydrate using increasing concentrations of ethanol - 95%, 100%, 100% (Fábrica do Álcool, Portugal; Proclínica, Portugal) (quick dip)
9. Diaphanization using xylene (2 minutes 2x)
10. Mount with Coverquick 2000 mounting media (VWR, France).

Appendix III

Technovit 7100 preparation

Dehydration

1. Ethanol 90% (Proclínica, Portugal) (1 hour)
2. Ethanol 96% ((Fábrica do Álcool, Portugal) (1 hour)
3. Ethanol 100% (Proclínica, Portugal) (1 hour)
4. Ethanol 100% (Proclínica, Portugal) (1 hour)

Pre infiltration

5. Prepare a solution using 100 ml of technovit and 1 gr of hardener I and shake for 10 minutes (infiltration solution)
6. Make a 1:1 dilution with ethanol 100% (the amount depends on the number of samples)
7. Embed the samples for 2 hours.

Infiltration

8. Overnight infiltration on the solution prepared in 5.

Polymerization

9. Mix 15 ml of infiltration solution and 1 ml of hardener II.

After preparing the support blocks with the solution prepared in 9 and the samples, leave it at room temperature for 1 hour and at 37ºC overnight.

Appendix IV

Haematoxylin and Eosin Staining for Technovit 7100 slides

1. Hydrate the slides with distilled water (5-10 minutes)
2. Stain with Mayer haematoxylin (Merck, Germany) (30 minutes)
3. Wash carefully with distilled water (5 minutes)
4. Staining with aqueous 1% eosin (Bio-Optica, Italy) (5 minutes)
5. Wash quickly and carefully with distilled water.
6. Dehydrate using increasing concentrations of ethanol - 95%, 100%, 100% (Fábrica do Álcool, Portugal; Proclínica, Portugal) (quick dip)
7. Diaphanization using xylene (2 minutes 2x)
8. Mount with Coverquick 2000 mounting media (VWR, France).

Appendix V

RNA quantification

1. Clean the place where the quantification will be performed with ethanol.
2. Clean the µDrop Plate (Thermo Scientific, USA) with water and ethanol.
3. Read the blanks - the same water used during samples' elution.
4. Put 2 µl of each sample to be read.

Note: Vortex and spin the samples before reading and clean the µDrop Plate between readings.

Appendix VI

cDNA synthesis

This reaction should be performed on ice for optimal results and according to the table below.

Table 3. Volumes (µl) needed for cDNA synthesis.

Component	**Volume (µl)**
iScript RT Supermix	4
RNA template (0,5 µg)	Variable
Nuclease-free water	Variable
TOTAL	**20**

The necessary volume of RNA template to have 0,5 µg of RNA is calculated bearing in mind the RNA quantification results, and can be obtained by this equation:

Q -------- 1000 µl
0,5 µg ------- x µl
x= (1000*0,5)/Q

Q – Concentration at 260 nm obtained in RNA quantification (µg/ml)

Note: Nuclease-free water and RNA volumes should be equal to 16 µl.
Once the Supermix, the RNA volume and the water are on the right tubes, it is time to incubate in a thermal cycler using the following protocol:

Table 4. Protocol for cDNA synthesis incubation.

Priming	5 minutes at 25ºC
Reverse Transcription	20 minutes at 46ºC
RT inactivation	1 minute at 95ºC

Appendix VII

Gradient Test

The protocol described below was the same to all the genes used in this study. The difference is only on the temperatures tested, which are described below the protocol.

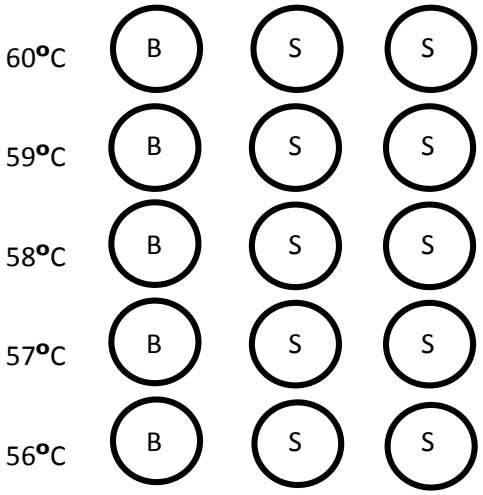
1. Pick five cDNA samples that were synthesised in double.

2. Prepare a mix of 2 µl of each sample with 40 µl of nuclease-free water – dilution 1:5.

3. Prepare a reaction mix with 10 µl of SYBR Green (Bio-Rad Laboratories, USA), 0,4 µl of each primer solution and 4,2 µl of nuclease-free water.

Note: These volumes are for each well needed. An additional volume must be prepared due to pipetting errors.

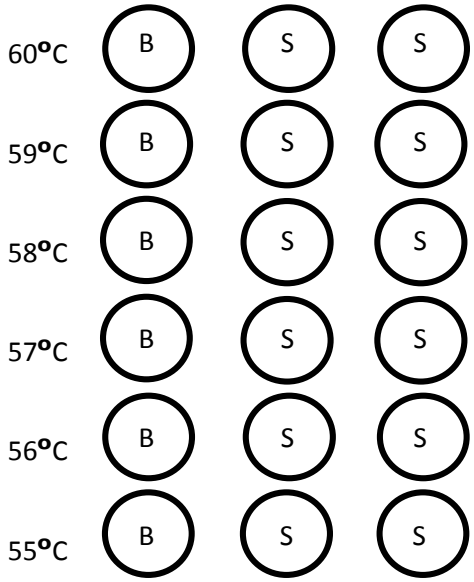
4a) Prepare a plate with 3 columns and 5 rows (for *acaa2*, *elovl1a, mapk1*, *lipca, myca* and *mycb* genes*)*



B – Blank (5 µl H_2O + 15 µl reaction mix)

S – cDNA sample (5 µl sample + 15 µl reaction mix)

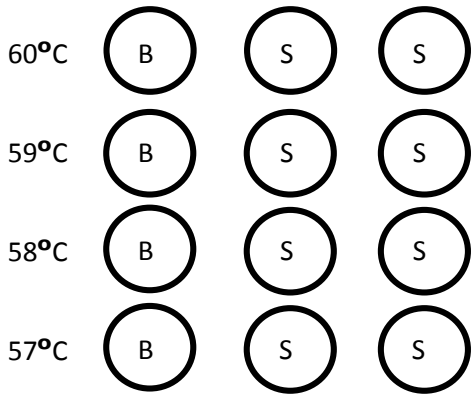
4b) Prepare a plate with 3 columns and 6 rows (for *p21*, *elfa1* genes*)*



B – Blank (5 µl H_2O + 15 µl reaction mix)

S – cDNA sample (5 µl sample + 15 µl reaction mix)

4c) Prepare a plate with 3 columns and 4 rows *(*for *tuba1*, *β-actin2*, *scd* genes*)*



B – Blank (5 µl H_2O + 15 µl reaction mix)

S – cDNA sample (5 µl sample + 15 µl reaction mix)

5. Read the plate and the best temperature is the one that has the lowest CT values.

Appendix VIII

Calibration Curve

1. Pick five samples that were synthesised in double during cDNA synthesis.

2. Prepare a mix of 1 µl of each sample with 15 µl of nuclease-free water – dilution1:5

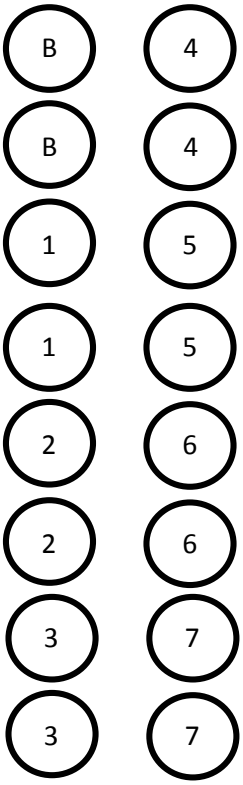
Note: these volumes must be altered if another dilution is required.

3. Prepare a reaction mix with 10 µl of SYBR Green (Bio-Rad Laboratories, USA), 0,4 µl of each primer solution and 4,2 µl of nuclease-free water.

Note: These volumes are for each well needed. An additional volume must be prepared due to pipetting errors.

4. Prepare 2 columns of a plate, as the example below shows.

5. Read the plate at the temperature chosen in gradient tests.



B – Blank (5 µl H_2O + 15 µl reaction mix)

1 – Standard 1 (5 µl sample + 15 µl reaction mix)

2…7 – Standards 2-7 (5 µl previous standard + 15 µl reaction mix)

Appendix IX

Samples analysis

1.Prepare a reaction mix with 10 µl of SYBR Green (Bio-Rad Laboratories, USA), 0,4 µl of each primer solution and 4,2 µl of nuclease-free water.

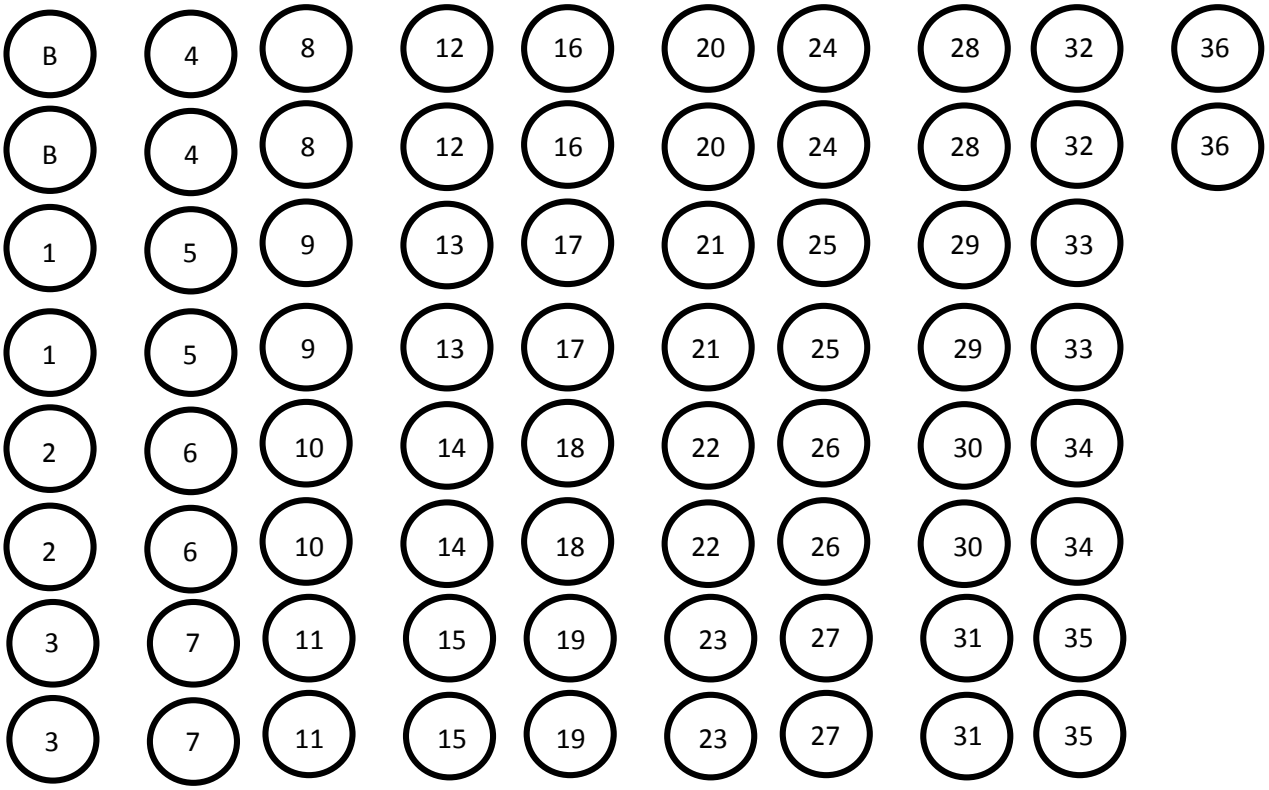
Note: These volumes are for each well needed. An additional volume must be prepared due to pipetting errors.

2. Pipette 15 µl of the previous prepared mix to each well of the plate.

3. Vortex and spin the samples.

4. Pipette 5 µl of the sample to the correspondent well.

5. Read the plate at the temperature chosen in gradient tests.



B – Blank (5 µl H_2O + 15 µl reaction mix)

1…36 – Samples (5 µl sample + 15 µl reaction mix)