Master Thesis

Characterization of the element of response to squalene in the murine gene *Cyp2b10*

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Abstract

The CYP2B10 gene possesses a role in the development of the liver disease, as it metabolizes ethanol, and this leads to the generations of reactive oxygen species (ROS) with the capacity to generate liver damage leading to liver disease. Other studies and research have found that squalene has protective role in terms of the liver disease and that it affects the expression of *Cyp2b10*. Therefore, the aim of this study is the characterization of *Cyp2b10* promoter, as well as the effects of squalene has on it. For the realization of this study, the promoter will be transfected into a line of AML12 (Alfa mouse liver cells 12) and then treated for 48 hours with different carriers of squalene. These carriers will be PLGA (poly (lactic-co-glycolic acid)), gelicelina and ethanol. Another component, lanosterol, will also be used, as a squalene metabolite of to see if the effect is due to squalene or its metabolite. Resulting from this study it has been found that squalene reduces the expression of *Cyp2b10*, that the best carrier out of the three studies is PLGA, followed by gelicelina and then ethanol.

Resumen

El gen CYP2B10 tiene un papel en el desarrollo de la enfermedad hepática, ya que metaboliza el etanol, y esto conduce a la generación de especies reactivas de oxígeno (ROS) con la capacidad de generar daño hepático que conduce a la enfermedad hepática. Otros estudios e investigaciones han encontrado que el escualeno tiene un papel protector en términos de la enfermedad hepática y que afecta la expresión de *Cyp2b10*. Por tanto, el objetivo de este estudio es la caracterización del promotor *Cyp2b10*, así como los efectos que tiene sobre él el escualeno. Para la realización de este estudio, el promotor se transfectará en una línea de AML12 (células hepáticas de ratón Alfa 12) y luego se tratará durante 48 horas con diferentes portadores de escualeno. Estos vehículos serán PLGA (poli (ácido láctico-co-glicólico)), gelicelina y etanol. También se utilizará otro componente, el lanosterol, como metabolito de escualeno para ver si el efecto se debe al escualeno o su metabolito. Como resultado de este estudio se ha encontrado que el escualeno reduce la expresión de *Cyp2b10*, que el mejor portador de los tres estudios es PLGA, seguido de gelicelina y luego etanol.

Abbreviations:

CYP: Cytochrome P450

AML12: Alfa mouse liver cells 12

ml/µl: millilitre/ microlitre

TE: Tris and EDTA

TBE X1: Tris-boric acid-EDTA

NaCl: Sodium chloride

LB: Luria broth

ADDE water: Distilled deionized water

DMEM: Dulbecco's modified Eagle medium

NEAA: Non-essential amino acid solution

ITS: Insulin-transferrin-selenium

EDTA: Ethylenediaminetetraacetic acid

PBS: Phosphate-buffered saline

PLGA: Poly (lactic-co-glycolic acid)

PPARβ/δ: Peroxisome proliferator-activated receptor β/δ

CAR: Constitutive active receptor

ROS: Reactive oxygen species

TxA_2: Thromboxane A_2

PGI_2: Prostacyclin

20-HETE: Hydroxyeicosatetraenoic acid

EETs: Epoxyeicosatrienoic acids

ADL: Alcoholic liver disease

Nfr2: Nuclear factor-erythroid 2-related factor 2

1- Background

1.1 Mediterranean diet

The Mediterranean diet has been associated with a reduction in the risk of suffering cardiovascular disease [1]. This is due to one of the main sources of fat in this diet, which is the virgin olive oil [2]. The way virgin oil acts and its effects as a protective mechanism are still in need to be elucidated. At first, the effects were attributed the oleic acid as the main component, with its minor bioactive components performing important biological actions [3], [4]. Squalene, which it was first isolated from shark liver oil [5], is a polyunsaturated triterpene that is a biochemical precursor of cholesterol that with its qualities has been proposed to be the relevant active compound rather than oleic acid [4].

While olive oil is the main fat source for the Mediterranean diet, is there is also a consumption of plant-based foods such as nuts, vegetables, legumes etc, dairy products primarily cheese and yogurt, as well as consumption of fish and poultry. Regarding meat, red meat is eaten sparingly. This provides a mixed nutrition that mixes undesirable saturated fatty acids and cholesterol with desirable nutrients such as fibres, monounsaturated fatty acids, complex carbohydrates, vitamins, etc [3].

1.2 Olive oil

In the Mediterranean diet, the main source of calories is the virgin olive oil, which is an oily matrix composed of hydrocarbons, with the main one being squalene, triterpenes like erythrodiol, uvaol, maslinic and oleanolic acids and a wide variety of different phenolic compounds such as phenols, secoiridoids, flavonoids and lignans [3].

1.3 Squalene

Squalene is a polyunsaturated triterpene, an organic compound that is produced by plants (like in olive oil) and animals (like in shark liver) as a biochemical intermediary, such as being a precursor of sterols in both plants and animals or being the precursor for cholesterol (Figure 1) and steroid hormones in humans [6]. Squalene has the important ability to reduce ROS damage to the skin or the rod receptors in the eye, it also possesses a detoxifying role for some xenobiotics, such as organochlorines, theophylline and strychnine [7].

Its contents in extra virgin olive oil can vary from 1.5 to 10.1 g kg-1 depending on variables such as the plant cultivar, the methods of processing of the fruit or the agronomical practices [8]. *In vitro* squalene is an effective oxygen scavenging agent [9] and remain stable at 180 degrees Celsius for 36 hours [10] making squalene the newly proposed relevant active compound in virgin oil [11].

In an study the intake of dietary squalene modified the lipoproteins on rabbit plasma provoking an increase in VLDL and LDL non-esterified cholesterols with an increase of hepatic nonesterified cholesterol, while in the liver cholesterol biosynthesis intermediates were modified [12] .

Another component that can have certain importance is lanosterol, which is a metabolite resulted from squalene, catalysed by CYPs, that eventually will lead down to cholesterol [13].

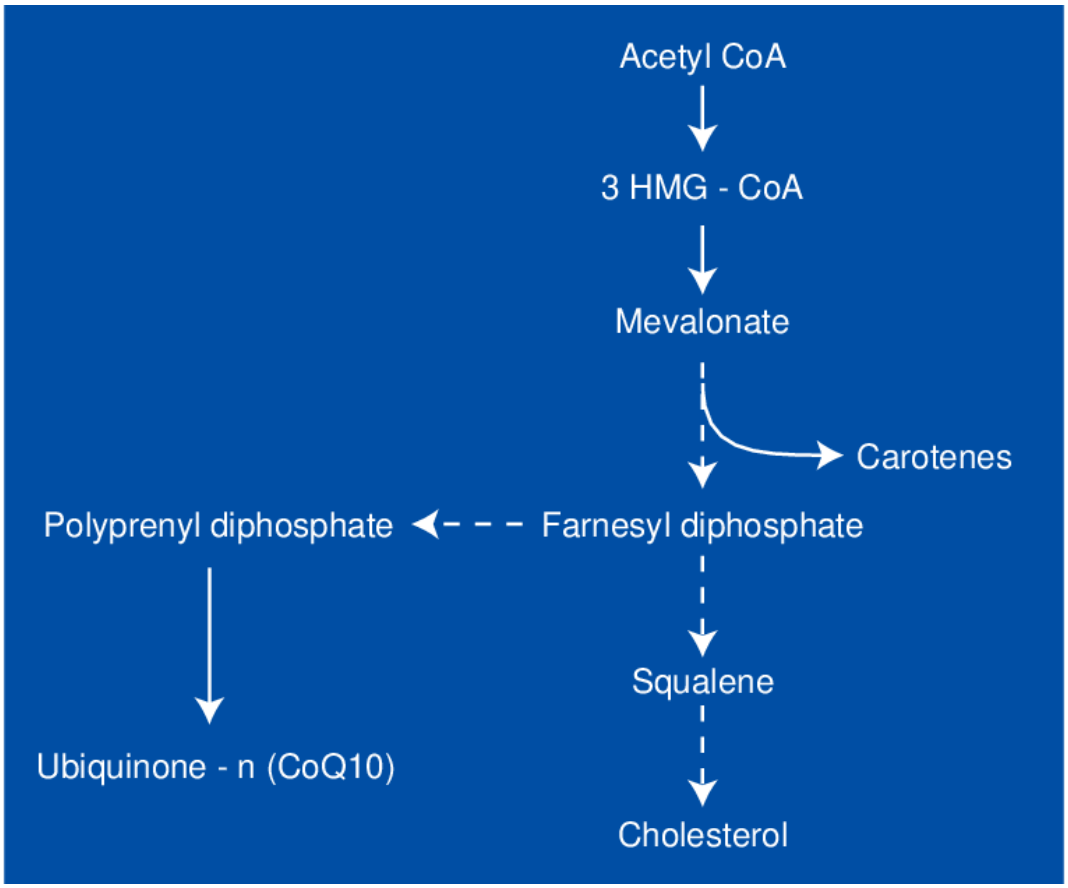

Figure 1- Squalene, carotene, cholesterol, and coenzyme Q10 synthesis [7]

1.3.1 Squalene effects on the liver

In the liver of *Apoe*-deficient mice, squalene administration upregulates the expressions of *Cyp2b10*, *Cyp2b9* and *Cyp2c55*. On a regime of 1 g kg-1 of squalene administration in a Western diet causes a decrease in hepatic triglycerides as well as in oxidative stress. The results were observed both in females and castrated male with exception of *Cyp2b9*. Mice that were receiving a diet with extra virgin oil also presented an increase in *Cyp2b10* and *Cyp2c55* leading to suggest that the squalene in the olive oil is the responsible for this effect [12].

1.4 Cytochromes P450

This superfamily is characterized for enzymes containing a heme group that acts as a cofactor with a monooxygenase function, and diverse reactions that CYPs catalyse, ranging from oxidations, peroxidation of xenobiotics, agrochemicals fatty acids, etc. In the case of mammals, these CYP oxidize steroids, xenobiotics, and fatty acids, they are also of importance in the clearance of several compounds and the synthesis and breakdown of hormones; they also partake in the synthesis of cholesterol. In plants, they are in charge of the biosynthesis of defensive compounds as well as fatty acids and hormones. In the case of bacteria, CYP is important for the role of several metabolic processes such as the biosynthesis of antibiotics [14], [15].

Organisms can produce many different CYPs, which with splicing capability produces a vast array of enzymes with different substrates. The nomenclature goes as follows, they have the CYP followed by a number designating the family, then a letter for the subfamily, then another number for referencing the protein. Furthermore, they can be grouped into two classes, class I for prokaryotic and mitochondrial and class II for eukaryotic microsomes; and there is also another naming scheme based on the number of components, such as class B for 3 components and class E for 2 components [14], [15].

1.5 Mechanism of action of CYPs

1.5.1 CYPs structure

As mentioned, cytochromes P450 has an active site that contains a heme-iron center, this iron is tethered via a cysteine thiolate ligand to the protein. The cysteine and some of the flaking residues in the region are highly conserved in the known CYPs This allows for a great variety of possible reactions to be catalysed via CYPs (Figure 2) [16].

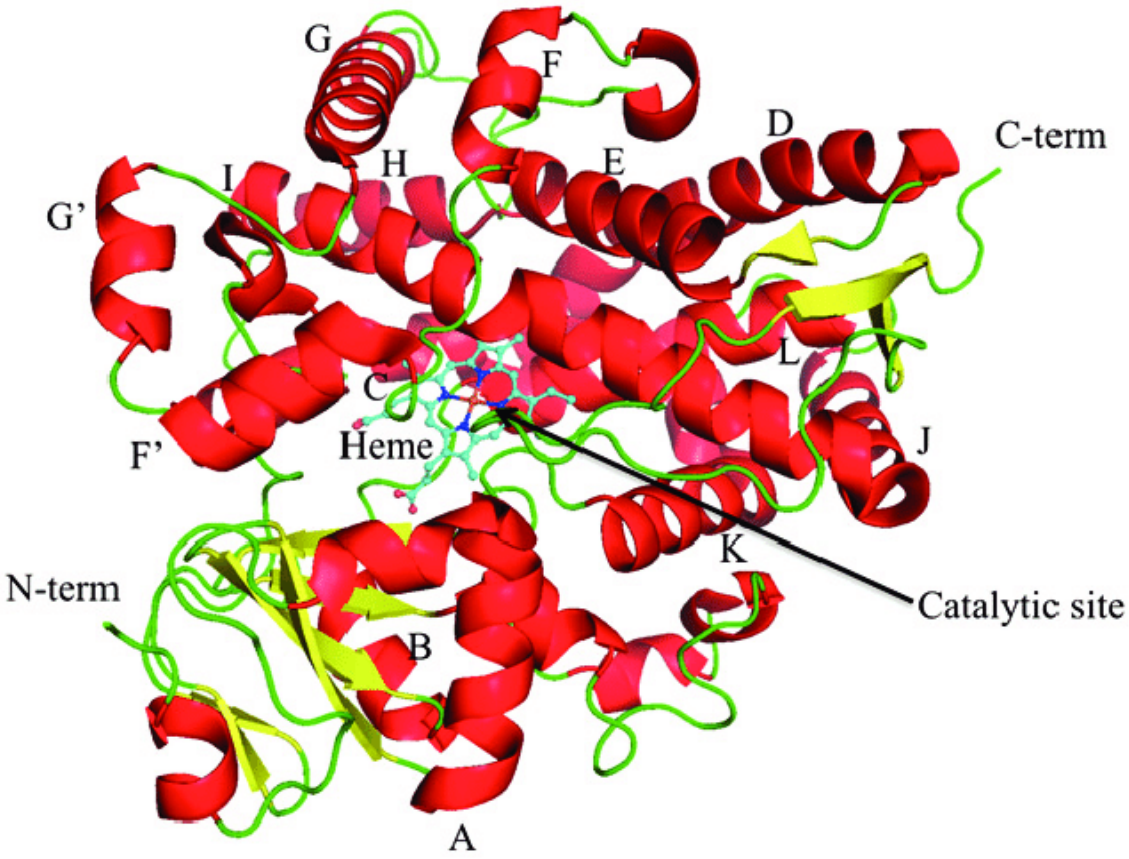

Figure 2- Structure of cytochrome P450 (CYP3A4) [17]

1.6 CYPs functions

Gene family	Number of genes	Main function
CYP1	3	Biotransformation
CYP2	16	Biotransformation
CYP3	4	Biotransformation
CYP4	12	Fatty acids metabolism
CYP5	1	Thromboxane A2 synthesis
CYP7	2	Bile acid biosynthesis
CYP8	2	Bile acid and prostacyclin biosynthesis
CYP11	3	Steroid biosynthesis
CYP17	1	Steroid biosynthesis
CYP19	1	Steroid biosynthesis
CYP20	1	Unknown
CYP21	1	Steroid biosynthesis
CYP24	1	Vitamin D degradation
CYP26	3	Retinoic acid hydroxylation
CYP27	3	Vitamin D3 and bile acid synthesis
CYP39	1	Cholesterol synthesis
CYP46	1	Cholesterol synthesis
CYP51	1	Cholesterol synthesis

Figure 3- Table of the different human CYP families with their associated function [18]

1.6.1 Drug metabolism in humans

CYPs are the primary enzymes involved in drug metabolism; they account for around 75% of the total metabolism and are located in the inner membrane of the mitochondria or in the endoplasmic reticulum. The majority of deactivation of drugs is directly or via excretion from the body. But there are also many that are bioactivated via CYPs such as the opiate codeine (Figure 4)[19].

The majority of the CYPs can metabolize more than one drug, at the same time one drug can be metabolized by different CYPs. There are many drugs that affect the activity of CYPs isoenzymes with positive or negative effects. The mechanism can be an enzyme induction via biosynthesis or direct inhibition of the enzyme's activity. An example of this process is phenytoin, an antiepileptic drug, that activates several CYPs, such as CYP1A2, CYP2C9, CYP2C19 and CYP3A4 [19].

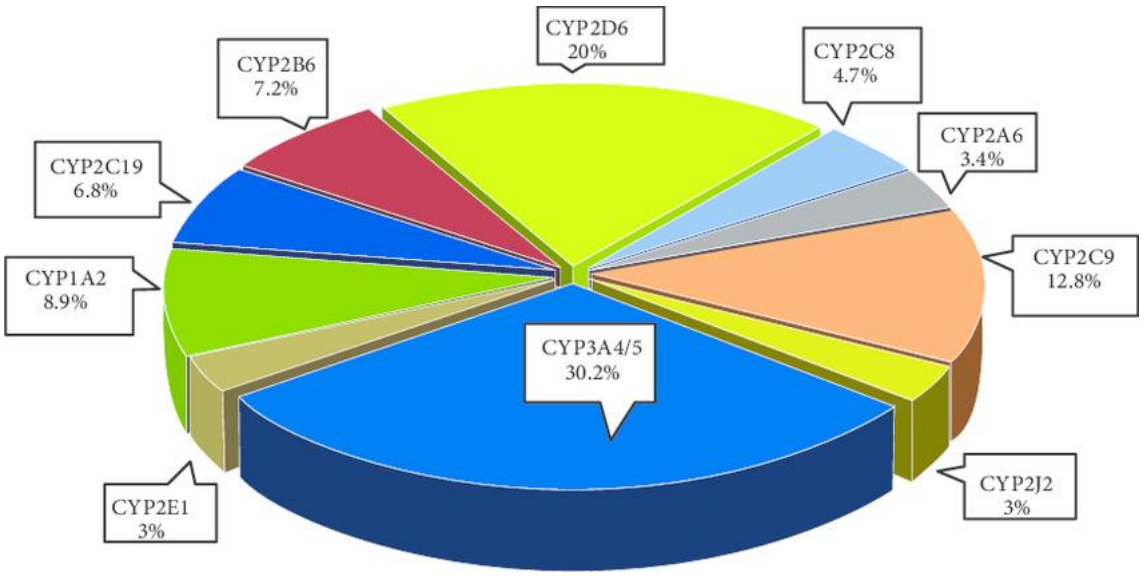

Figure 4- Percentage of all prescription drugs metabolized in human liver by a particular CYP enzyme [20]

1.6.2 Lipid metabolism

The CYPs are involved in different processes such steroid biosynthesis, where CYP11A1 is the first enzyme involved in the steroidogenesis pathway (Figure 5) making it essential for the synthesis of glucocorticoids, mineralocorticoids and sex steroids, with other CYPs being involved, such as CYP17A1 for the first two and CYP19A1 for the synthesis of estrogens [21].

Another process is the fatty acid metabolism, where the CYP4 family is mainly involved in the biosynthesis of fatty acid derivatives, with an example CYP4A11 oxidizing lauric acid or CYP4F2 and CYP4F3 that oxidise the omega position of very long fatty acid chains [22].

Cholesterol synthesis where CYPs like CYP17 are involved in the metabolic pathway for the synthesis of cholesterol (Figure 5) [23].

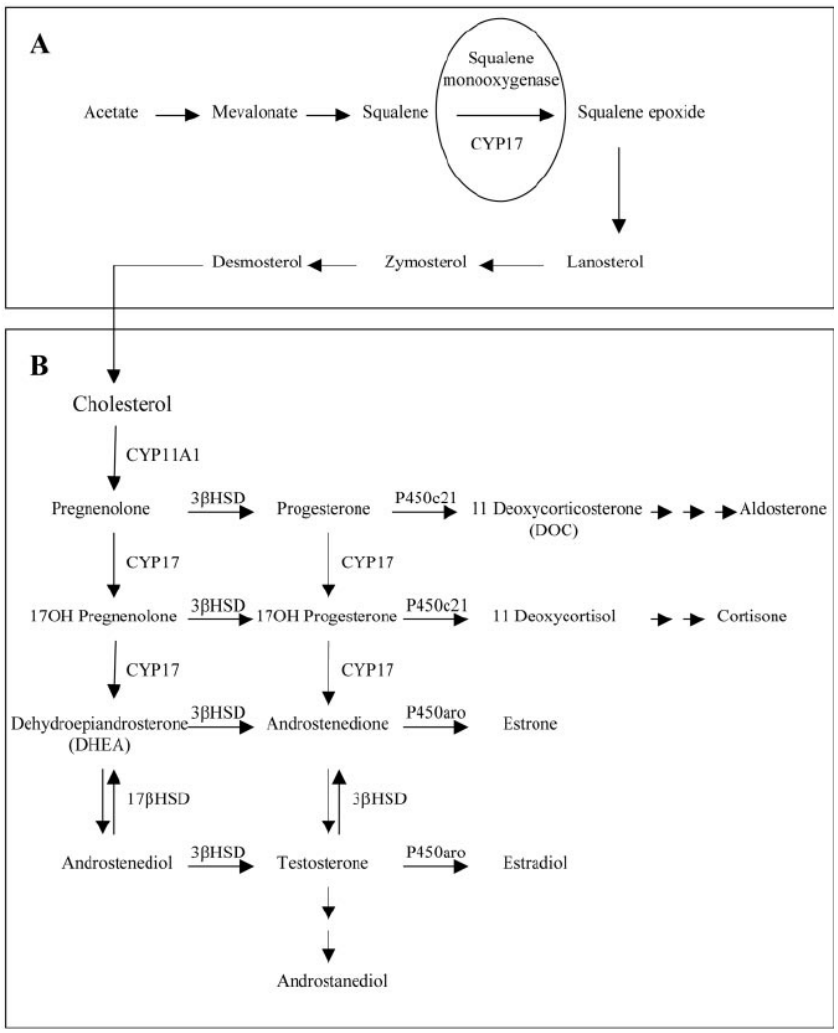

Figure 5- Cholesterol Biosynthesis (A) and Steroidogenic Pathway (B) Each arrow represents one or more enzymatic steps in cholesterol and steroid biosynthesis [23] **.**

1.7 CYPs in other species

While humans possess 57 genes for CYPs and more than 60 pseudogenes, animals have a wide range, from 35 genes in the sponge *Amphimedon queenslandica* to 235 genes in the cephalochordate *Branchiostoma floridae* [10].

CYPs have been researched in mice, rats, dogs, in order to ease the use of these model organisms for drug discovery and toxicology [24]. Recently CYPs have also been discovered in avian species, in particular turkeys, that may turn out to

be a useful model for cancer research in humans. CYP1A5 and CYP3A37 in turkeys were found to be very similar to the human CYP1A2 and CYP3A4 respectively, in terms of their kinetic properties as well as in the metabolism of aflatoxin B1[25]. CYPs have also been studied in insects, often to understand pesticide resistance. For example, CYP6G1 is linked to insecticide resistance in DDT-resistant Drosophila melanogaster and CYP6M2 in the mosquito malaria vector *Anopheles gambiae* is capable of directly metabolizing pyrethroids [26].

1.8 Important CYPs families and their obligation in the Human

The human genome project has identified 57 different human's CYPs, divided into 18 families and 43 subfamilies using sequence similarity for the classification [27]. The families that are responsible for the metabolism of drugs, xenobiotics and some endogenous molecules are families CYP1, CYP2 and CYP3.

1.8.1 CYP1A

The CYP1A family possesses two enzymes CYP1A1 and CYP1A2. Their position on the genome is on chromosome 15 q24.1. The reactions catalysed are the hydroxylation and oxidation of aromatic compounds with CYP1A1 metabolizes aromatic carbons, CYP1A2 metabolizes aromatic amines and heterocyclic compounds [28].

While CYP1A1 does not have a significant expression in the liver, it can be found in the lungs, mammary glands, placenta, and lymphocytes [29]. Meanwhile CYP1A2 is primarily expressed on the liver [30].

1.8.2 CYP2D

The CYP2D subfamily is primarily represented by CYP2D6, this enzyme metabolizes a large number of drugs, with importance in the breakdown of analgesics such as tramadol and codeine [31]. More than 70 polymorphisms of this enzyme have been catalogued, with most of them having poorer metabolically performance, with some cases where genotypes present gene duplication that provokes a rapid metabolization which can lead to opiates related side effects [32].

1.8.3 CYP3A

The CYP3A4 is one of the most important in the CYP3A family, being the most common and versatile. It is located on chromosome 7q22.1. It's involved in catalysis of many reactions in drug metabolism, synthesis of cholesterol, steroids, and other lipid components with its monooxygenase capability. It is located on the endoplasmic reticulum and can be induced by glucocorticoids and other pharmacological agents [33].

1.8.4 Other CYPs families

There are other families with many more diverse roles, but a couple of example of families that are related to the metabolism of cholesterol is CYP51 that has a role on its biosynthesis, while the CYP7A1, CYP27A1, CYP7B1 and others are vital on the catabolism of cholesterol to form bile acids [34].

1.8.5 CYP2B

The CYP2B subfamily contains enzymes that are expressed in lung and liver tissues of different animals, this includes mouse CYP2B10 or human ortholog CYP2B6 (with the position on the human genome on chromosome 19q13.2), rats with CYP2B1, rabbit with CYP2b4, etc [35].

The CYP2B family is able to metabolize a great range of different endogenous, pharmaceutical and environmental agents, therefore, this polymorphic capacity should be taken into account at the time when evaluating pharmaceutical designs or the susceptibility to certain environmental toxicants [36].

In the lungs of rabbit, the vast majority of P450 enzymes are 2B4 and 4B1, which both catalyse the bioactivation of 4-ipomeanol. *Cyp2b10* is expressed in the mouse Clara cells and alveolar epithelial cells at high levels, there is also detection of this enzyme in the liver, but only after an induction via phenobarbital. Rats on the other hand present two isoforms, CYP2B1 predominant of the lung and CYP2B2 which is predominant in the liver [35].

The human isoform CYP2B6 has been identified in both liver and lungs and has been demonstrated that be one of the most abundant P450 mRNAs that have been observed in the lungs [37]. CYP2B6 is also highly polymorphic, with almost one-hundred different forms between individuals, and these differences were not only based in race but also in gender, with women possessing nearly two times more than males [38].

1.9 CYP2B10

This is the CYP2B isoform present in the mouse, present primarily on the adult duodenum and adult lungs, reduced expression in the adult liver and small intestine, and minimal expression in the large intestine and adult ovaries. On the genome, it is found on the 7th chromosome, the location being 25,597,083-25,626,049. It possess 9 exons (Figure 6) [39].

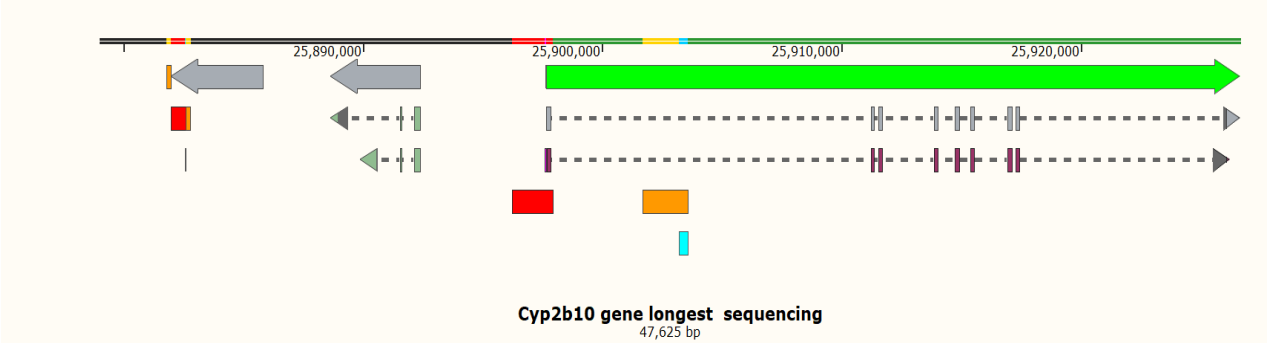

Figure 6 – *Cyp2b10* gene sequencing, took with SnapGene

Some of the functions of CYP2B10 are a pentoxyresorufin O-deethylase activity, that is expressed in the livers of all strain mice and sexes and is induced by phenobarbital. Hepatic CYP2B10 has been proposed to also metabolize testosterone to 16α- hydroxytestosterone [12]. Another function of CYP2B10 is the mediation through its alcohol dehydrogenase activity on the metabolism of ethanol [40].

1.9.1 CYP2B10 and squalene

It has been shown that a dose of 1 g/kg of squalene caused a decrease in ROS in lipoprotein fractions, with the independence of the background of the animal, which provoked an increase in high-density lipoproteins levels. This was accompanied by a decrease of ROS in lipoprotein fractions, though these effects are dependent on the presence of apolipoprotein A1 [41].

Administration of squalene in doses of 1 and 0.25 g/kg in the diet chow induced, in both wild type and apolipoprotein E deficient mice, the expression of *Cyp2b10* and *Cyp2c55* and with the independence of the dosage. Moreover, the incubation of squalene also provoked the expression in HepG2 cells of *CYP2B6* the human ortholog of CYP2B10. Thus, this demonstrates that squalene has CYP2B10 and CYP2C55 as targets with no dependence on a diet. With squalene providing a protective effect against the ROS even though there has been an increase of CYP2B10 that can generate more ROS, attributed to the antioxidant induction via squalene [12].

1.10 Diseases related to CYPs

1.10.1 Atherosclerosis

The primary cause of coronary, cerebral and peripheral diseases is atherosclerosis [42]. It involves a chronic and progressive vascular inflammation that is accompanied by endothelial dysfunction, dyslipidaemia and platelet hyperactivity [43]. There are some lipids that play an important role in terms of modulating atherosclerosis, those being thromboxane A_2 (TxA_2) and prostacyclin

(PGI_2). [44] The CYP5A1 and CYP8A1 are involved in their synthesis. the mutation of these CYP genes can result in the dysfunction of the gene, leading to a negative impact on platelet aggregation atherogenesis, bone density hypertension and altered vasodilation [45], [46].

1.10.2 Renovascular diseases

Renal homodynamic is regulated by hydroxyeicosatetraenoic acids (20-HETE) and epoxyeicosatrienoic acids (EETs). With 20-HETE being a powerful vasoconstrictor and acting as an inhibitor for sodium transport in the loop of Henle and proximal tubule, and with EETs act in the collecting duct and proximal tubule to dilate small arterioles and to block the sodium transport. Thus the more 20-HETE there is an increase in the pressor actions leading to a more acute and chronic renovascular diseases [47]. The CYP2J2, CYP epoxygenases, and CYP-ω hydroxylases have a role in their synthesis [48]. Alteration of these CYPs have been reported to modulate renal injury pathogenesis and hypertension, such as renovascular disease and also related to increased cardiovascular mortality [49], [50].

1.10.3 Ischemic heart disease

The CYP that have a role in this disease are C2P2C and 2J families, which are epoxygenases. They take part in the conversion of arachidonic acid into epoxyeicosatrienoic acids (EETs) in the cardiovascular tissue. These EETs have different effects that are protective such as vasodilatory, antithrombotic, antiapoptotic and anti-inflammatory actions [51].

1.10.4 Liver disease

One type of liver disease is alcoholic liver disease (ADL), which is caused by abuse in the ingestion of alcohol, and leads to either a fatty liver, liver inflammation or liver fibrosis and cirrhosis. CYP2E1 is expressed in multiple organs but primarily in the liver, can be induced by different conditions such as obesity, diabetes, alcohol consumption, high fats diets, etc, which leads to being that CYP2E1 is a major risk factor for liver disease [52].

Another CYP involved in the liver is CYP2A5, which is also induced by alcohol consumption, but this induction is dependent on CYP2E1. CYP2E1 generates ROS, which in turn upregulates nuclear factor-erythroid 2-related factor 2 (Nfr2), which provokes an enhancement in CYP2A5, but it does not promote ADL, rather the opposite giving a protective effect [53], [54].

1.10.5 CYP2B10 and liver disease

CYP2B10 with CYP2E1 mediate through their alcohol dehydrogenase activity the metabolism of ethanol [40]. The oxidation that the ethanol suffers generates highly reactive oxygen species (ROS) with the capacity to cause liver damage, and with enough damage to liver disease [55]. Therefore, the alteration in the expression of these enzymes has an impact on ethanol consumption sensitivity. [40].

PPARβ/δ, the peroxisome proliferator-activated receptor- β/δ, is able to protect the liver from chemical injuries with mechanisms such as inhibition of steatosis and inhibition of inflammation [56], [57]. While it is thought that CAR activation mediates the expression of CYP2B10 [58], there are some studies that show that an ethanol-induced expression of CYP2B10 mediated by PPARβ/δ that did not influence the observed expression of CAR [59], [60], though the ligand activation of CAR provoked and up-regulation of CYP2B10 in PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ in mouse liver [59].

2-Objectives

The objectives can be divided into four different aspects:

- Characterization of the DNA element in the promoter that response to squalene in the murine *Cyp2b10* gene.
- To compare different carriers to increase the efficiency of squalene delivery
- Study the effect of squalene in comparison with lanosterol on *Cyp2b10* promoter activity
- To investigate the expression level of *Cyp2b10* in presence of squalene

3- Materials and methods

3.1 Generation of reporter gene constructs

3.1.1 Construction of pEZX-GA01 with *Cyp2b10* promoter

The genomic region of the *Cyp2b10* promoter (Figure 7) (2025 bp long), was inserted into the pEZX-GA01 multicloning site (XhoI- BglII) with the primers forward 5' CCAGATCTTGGAATTGTGGCCATCAAGAGCTTGGC 3' and reverse 5' GATATCTGCAGAATTATCTTTTATGCTGGCCCCGC 3' upstream of a secreted Gaussia luciferase (GLuc) reported gene using linearized pEZX-GA01 (Genecopoeia, Rockville, MD) according to the In-Fusion cloning protocol from Takara-Clontech (cat. no. 638909; Kusatsu, Shiga, Japan). Restriction enzymes and DNA sequencing confirmed the resulting plasmid.

The genomic region of the *Cyp2b10* 350 bp promoter fragment was inserted into the pEZX-GA01 multicloning site (HindIII- BglII) with the primers forward 3' CCAGATCTTGGAATTAGACACAGAAATACACATCCGTAC 5' and reverse 3' GATATCTGCAGAATTATCTTTTATGCTGGCCCCGC' 5' upstream of a secreted Gaussia luciferase (GLuc) reported gene using linearized pEZX-GA01 (Genecopoeia, Rockville, MD) according to the In-Fusion cloning protocol from Takara-Clontech (cat. no. 638909; Kusatsu, Shiga, Japan). Restriction enzymes and DNA sequencing confirmed the resulting plasmid.

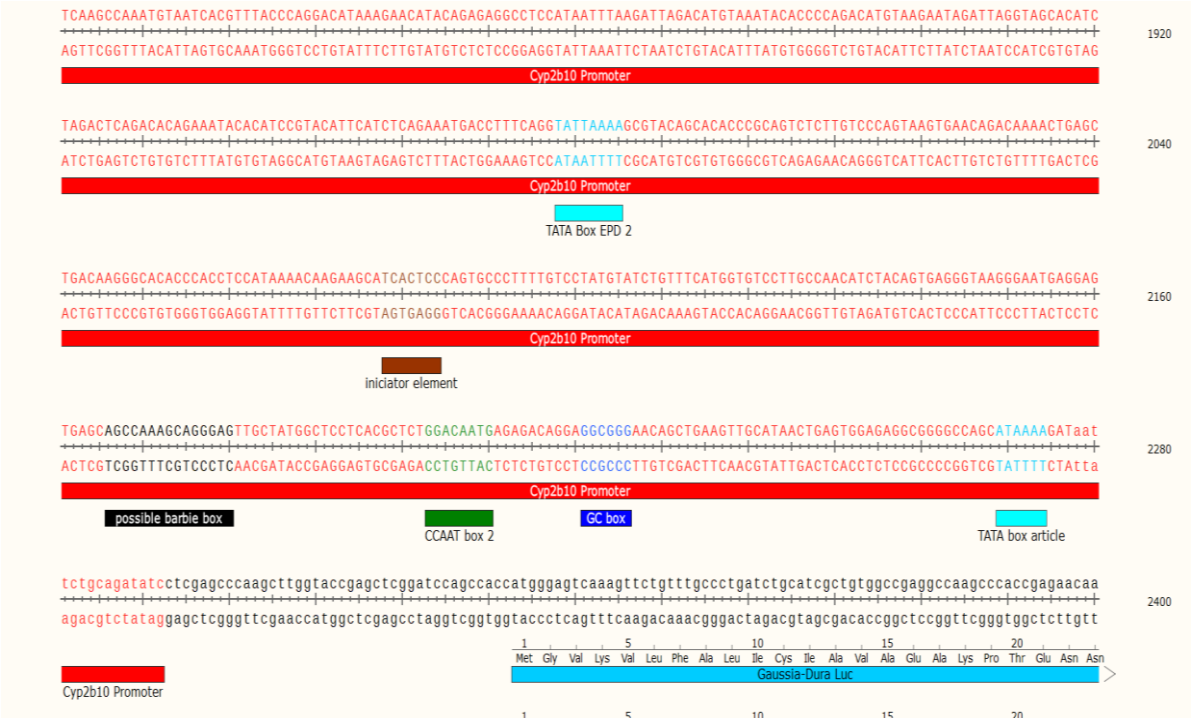

Figure 7- Final sequences of the *Cyp2b10* promoter with the important motifs: TATA box (Light Blue), GC box (Dark Blue), CCAAT box (Green), barbie box (Black) initiator element (Brown)

In order to choose the primers and sequences for the experiments several web sites and information repositories where used, such as ensemble.org, NCBI and Takarabio. Another program used was SnapGene, that was used to visualize the whole fragment and delimit the important sequences such as primers to use, TATA box, etc.

The plasmid pEZX-GA01 (Genecopoeia, Rockville, MD) (Figure 8) is composed of the cytomegalovirus promoter (position 869-1378) used as an immediate-early enhancer and promoter, there are also the gene reporter sequences to check that the plasmid and the gene promoter have been transferred and integrate correctly, these are the alkaline phosphatase (position 1442-2986) and the luciferase (position 8-565) that act as reporter genes in order to detect and measure the gene expression.

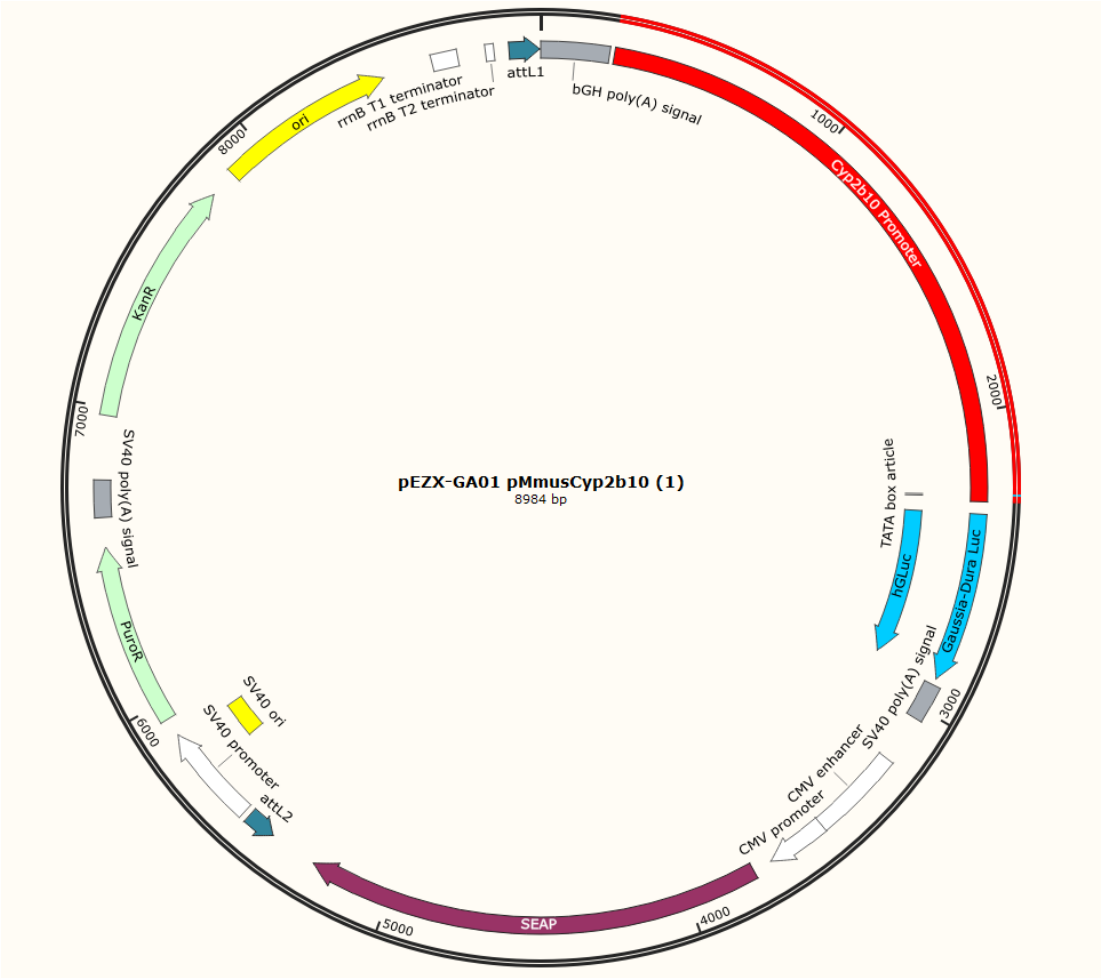

Figure 8- pEZX-GA01 plasmid use for the experiment, with the different sequences it holds.

PCR is made with the extracted samples in order to confirm the correct insertion of the promoter (Taq buffer 2 µl, $MgCl_2$ 1.6 µl, Taq enzyme 0.125 µl, primer Forward 5' CCAGATCTTGGAATTGTGGCCATCAAGAGCTTGGC 3' and primer Reverse 5' GATATCTGCAGAATTATCTTTTATGCTGGCCCCGC 3' for the full promoter and forward 3' CCAGATCTTGGAATTAGACACAGAAATACACATCCGTAC 5' and reverse 3' GATATCTGCAGAATTATCTTTTATGCTGGCCCCGC' 5' 0.5 µl, DNA 1 µl, dNTPs 0.2 µl and H_20 14.075 µl per each sample)

3.2 Cell culture

The murine hepatocyte cell line was grown in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's minimum essential medium (DMEM) (ThermoFisher Scientific): F12-Ham's medium (GE Healthcare Life Science, South Logan, Utah) supplemented with foetal bovine serum and insulin/transferrin/selenium. When AML12 cells reached a 90–100% confluence, this medium was removed, then added the medium devoid of foetal bovine serum. Cells were incubated for 6 h in the presence of 200 nM lanosterol (Avanti Polar lipids, Alabaster, AL, USA) dissolved in 0.1% methanol. For other conditions, instead of adding the lanosterol, the cell is incubated in the presence of squalene 15 µM, 30 µM and 50 µM dissolved in 0.1% ethanol respectively, squalene 15 µM, 30 µM, 50 µM and 100 µM dissolved in 0.1% Gelicelina (made from choline and glycerol by Department of Physical Chemistry at Zaragoza University) respectively, or squalene 15 µM, 30 µM and 50 µM in PLGA (made of PLGA, Pluronic F68, ethyl acetate, and dH_2O, sonicated by 80% amplitude for 25 seconds, then evaporation step for 3h, 600 rpm and final step was centrifugation 15000g 15 min, 10 degree) respectively.

3.3 Transfection and luciferase and alkaline phosphatase assays

Transfections of cells were performed by lipofectamine 3000 transfection kit (Invitrogen). Twenty-four hours upon transfection, cells were stimulated for 48 h with the different components previously mentioned. The luciferase activity generated in cell cultures was assessed using a coelenterazine buffer (Medchemexpress) 6 µM on a FluoStar Omega at a 500 nm wavelength. Results are expressed as relative light units (RLU).

A prior measurement of alkaline/phosphatase activity was made in a Spectro Nano Star device. Using a buffer (4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) in a diethanolamine buffer) in a spectrophotometer SpectroStarNano.

3.4 RNA isolation

RNA was isolated from each liver using Tri-reagent (Ambion, Austin, TX, USA). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from Ambion. RNA was quantified by absorbance at $A_{260/280}$. The integrity of the 28*S* and 18*S* ribosomal RNAs was verified by agarose gel electrophoresis and the 28*S*/18*S* ratio was greater than 2. RNA quality was also tested using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 nano kit, Santa Clara, CA, USA) and the RNA integrity numbers were higher than 8.2.

3.5 Quantification of mRNA

The potential changes in *Cyp2b10* (forward 5' TGCTGTCGTTGAGCCAACC 3' and reverse 5' CCACTAAACATTGGGCTTCCT 3') mRNA expression was determined by RT-qPCR analysis of individual samples using equal amounts of DNA-free RNA from each condition. First Strand cDNA synthesis kit (Thermo Scientific) was used to generate the complementary DNA. RT-qPCR reactions were performed using the Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers were designed using Primer Express® (Applied Biosystems) and checked by BLAST analysis (NCBI) to verify gene specificity as well as to get amplification of the cDNA but not genomic DNA. RT-qPCR reactions were performed in a Step One Real Time PCR System (Applied Biosystems) following the standard procedure. The relative amounts of all mRNAs were calculated using the comparative $2^{-\Delta\Delta Cq}$ method and normalized to the reference expression of *Tbp* (using direct 5' GTGAGTTGCTTGCTCTGTGC 3' and reverse 5' GCTGCGTTTTTGTGCAGAGT 3'*)* and *Ppib* (direct 5' GGAGATGGCACAGGAGGAA 3' and reverse 5' TAGTGCTTCAGCTTGAAGTTCTCAT 3') in AML12 samples.

3.6 Statistical analysis

Results are presented as means and their standard deviations. The normal distribution of data was analyzed according to Shapiro–Wilk test, and homology of variance among groups using Bartlett's or Levene's tests. Parameters fitting both criteria were analyzed using 2-tailed Student's *t* test or one-way ANOVA with Bonferroni's multiple-comparison test as post hoc analysis. A Mann-Whitney *U* test or non-parametric Kruskal–Wallis ANOVA followed by Dunn´s multiple comparisons were used to compare the groups failing in any of the hypotheses. All calculations were performed using GraphPad Prism 5 for Windows (GraphPad, S. Diego, CA, USA). A *P* value of less than 0.05 was considered statistically significant.

4- Results

4.1 Characterization of the element of response to squalene in the mouse *Cyp2b10*

Eukaryote Promoter Database (EPD) was used for the characterization of the *Cyp2b10* promoter in order to find and delimit the important sequence of the promoter. The result showed that the promoter (bp -1 to -350) contains most of the important motifs such as the TATA box, GC box, CCAAT box, and the initiator element (Figure 9).

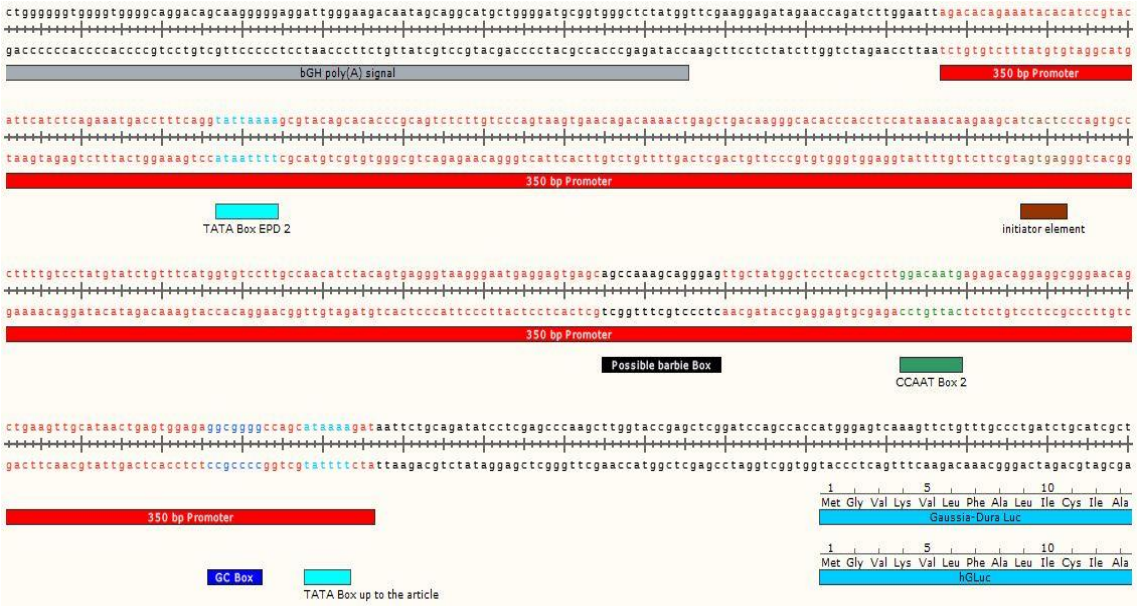

Figure 9- *Cyp2b10* 350bp promoter sequence with the important motifs: TATA box (Light Blue), GC box (Blue), CCAAT box (Green), barbie box (Black) initiator element (Brown)

To evaluate the function of the new promoter segment, we examined the activity of luciferase referred to alkaline phosphatase in AML12 cells and compared it to the full promoter. The result, shown in Fig. 10, indicated that the activity of the 350 bp promoter is increased by 41%.

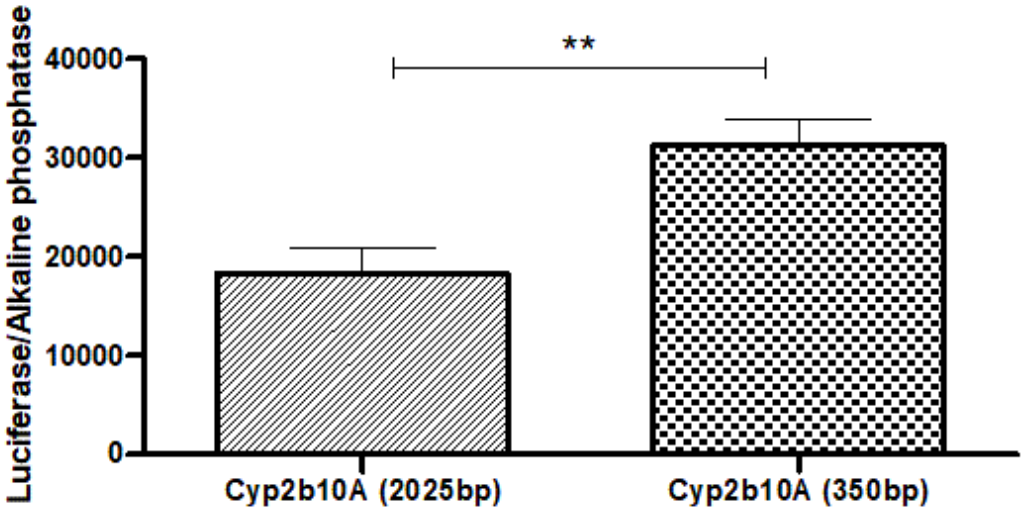

Figure 10 Difference of the *Cyp2b10* promoter activity

Comparing the full *Cyp2b10* promoter against the 350 bp fragment. (, P value = 0.0007)**

For investigating the squalene repercussion on C*yp2b10* promoter, activity of luciferase referred to alkaline phosphatase was evaluated in presence of 30 µM of squalene and the results illustrated that the squalene induces 350 bp *Cyp2b10* promoter more than the full promoter but there was not a significant difference (Figure 11).

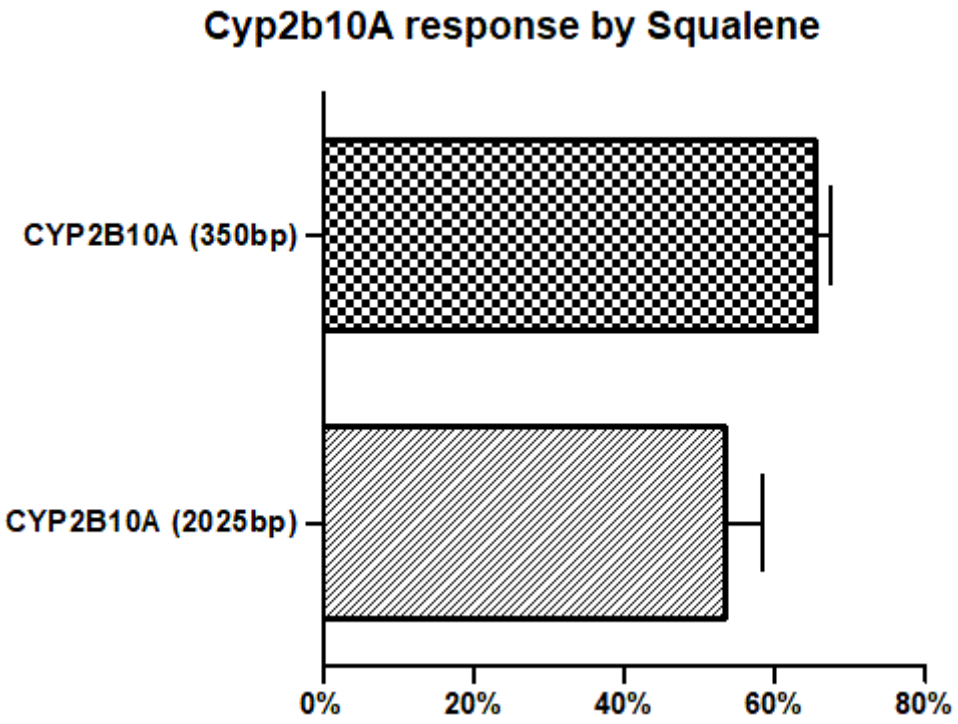

Figure 11 Difference of the *Cyp2b10* induction by squalene dissolved in PLGA

4.2 C*yp2b10* promoter activity under squalene influence

The figure 12 reveals that the squalene based PLGA nanoparticles decrease the *Cyp2b10* promoter activity, furthermore, this reduction in 350 bp of the promoter is higher than the full promoter. Besides, the most efficient concentration of squalene encapsulated with PLGA is 30 µM which had a higher decrement in comparison to other concentrations.

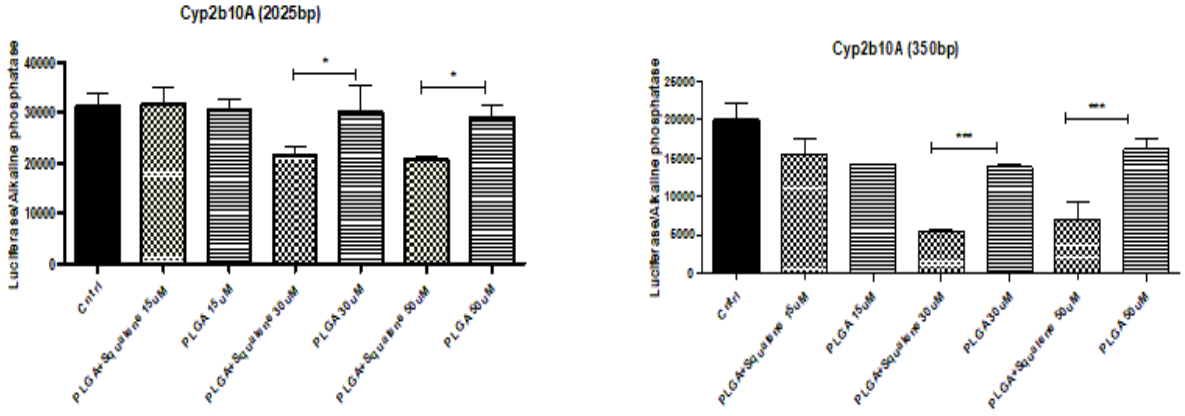

Figure 12 A) Comparing the difference of promoter activity of C*yp2b10* 2025 bp of different concentration of squalene with PLGA B) Comparing the difference of promoter activity of *Cyp2b10* 350 bp with different concentrations of squalene with PLGA

Comparing the full C*yp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value = ≤ 0.05) Comparing the full C*yp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value ≤ 0.05) Comparing the 350 bp C*yp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value = ≤ 0.0005) Comparing the 350 bp C*yp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value = ≤ 0.0005)

4.3 Different carriers and the squalene efficiency

To measure the squalene effect, we transferred the squalene to the AML12 cells with different carriers such as ethanol, PLGA, and Gelicelina and evaluated the *Cyp2b10* full and 350 bp promoter. Ethanol as a common carrier for squalene did not change the *Cyp2b10* promoter at 30 µM concentration in both promoter segments, in spite, there was a slight reduction in squalene sample, but this reduction was not significant (Figure 13).

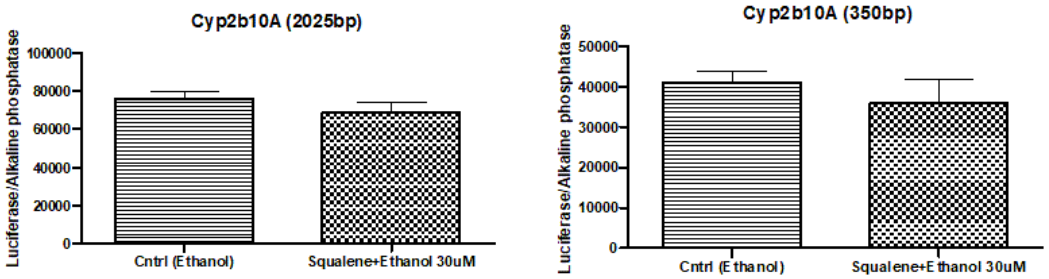

Figure 13- A) Comparing control of the full promoter C*yp2b10* against the ethanol squalene at 30 µM B) Comparing control of the 350 bp promoter *Cyp2b10* against the ethanol + squalene at 30 µM

Squalene-based PLGA nanoparticles showed a significant induction on both *Cyp2b10* fragments and in comparison, with ethanol which had a 12% reduction, presented a 60% of decline (Figure 14).

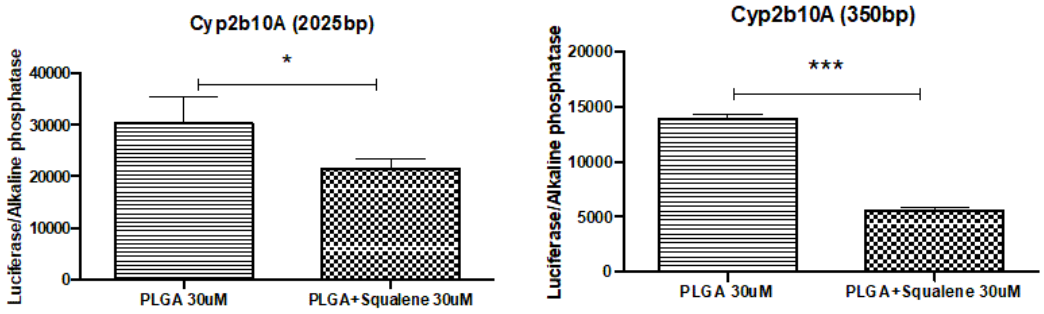

Figure 14- A) Comparing control of the full promoter *Cyp2b10* against the PLGA + squalene 30 µM B) Comparing control of the 350 bp promoter *Cyp2b10* against the PLGA + squalene 30 µM

Comparing the full *Cyp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value = ≤ 0.05)
Comparing the 350bp *CYp2b10* promoter 30 µM PLGA+ Sq against 30 µM. (P value = ≤ 0.0005)

To explore the squalene efficiency with gelicelina carrier, results claimed that the 30 µM concentration of squalene was not enough to have a considerable diminution in full *Cyp2b10* promoter and even in 350 bp fragment. In consequence, promoters have been exposed to higher concentrations of squalene with gelicelina and the outcome discloses a huge induction by squalene encapsulated with gelicelina.

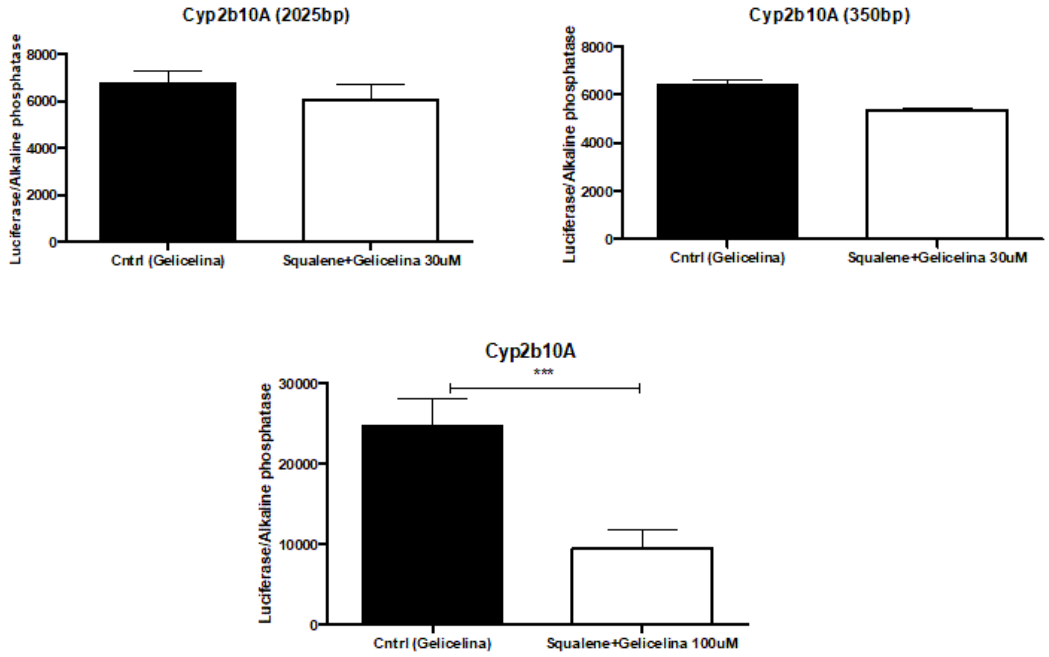

Figure 15- A) Comparing control of the full promoter C*yp2b10* against the Gelicelina + squalene 30 µM B) Comparing control of the 350 bp promoter *Cyp2b10* against the Gelicelina + squalene 30 µM C) Comparing control of the full promoter *Cyp2b10* against the Gelicelina + squalene 100 µM Comparing the full *Cyp2b10* promoter 100 µM Gelicelina+ Sq against control. (P value = ≤ 0.0005)

4.4 Effect of squalene in comparison to lanosterol in *Cyp2b10* promoter activity

To evaluate the effects of lanosterol compared to the effects of squalene on promoter activity, an experiment with lanosterol at different concentrations was carried out (Figure 16). While there was a small decrease at the 200 nM concentration of lanosterol, it was not significant.

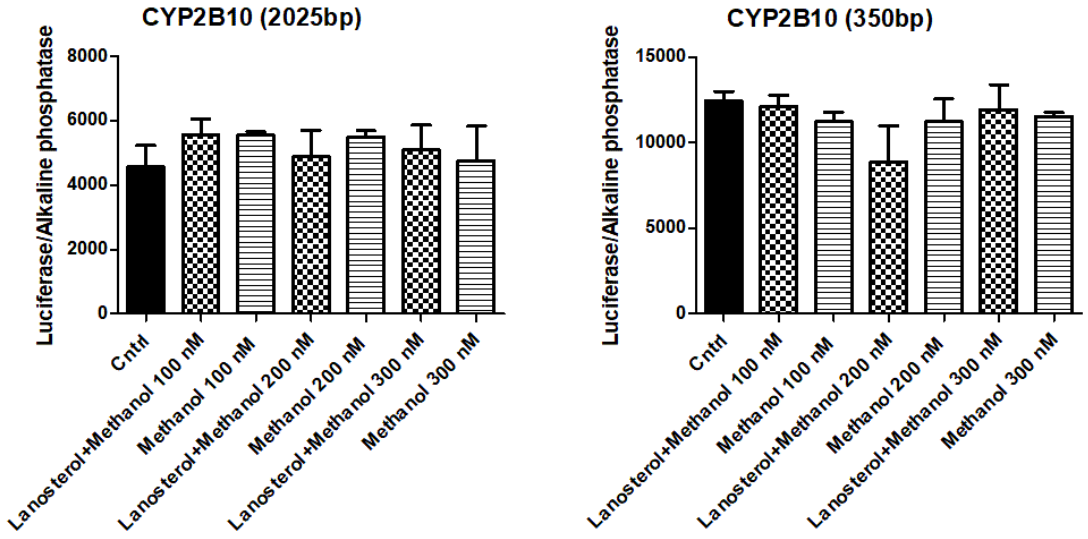

Figure 16- A) Comparing the difference of promoter activity of *Cyp2b10* 2025 bp of different concentration of lanosterol B) Comparing the difference of promoter activity of *Cyp2b10* 350 bp with different concentrations of lanosterol

4.5 Expression level of *Cyp2b10* in presence of squalene

The mRNA expression of the *Cyp2b10* gene has a significant change in presence of squalene and its expression has reduced when it was treated with squalene via PLGA and Gelicelina. The result of *Cyp2b10* expression in presence of 100 uM of squalene with gelicelina and 30 uM of squalene with PLGA are shown in figure 17.

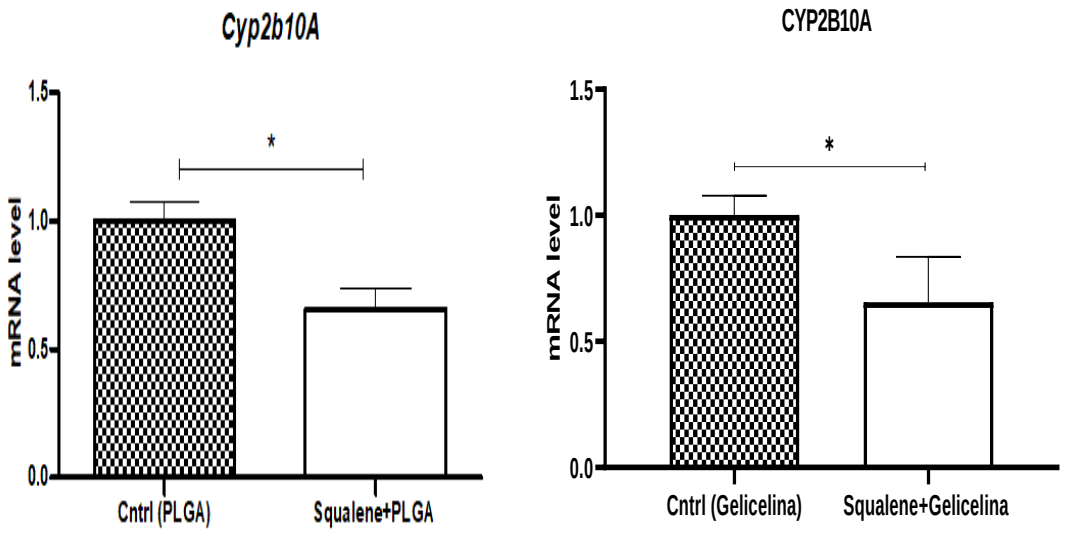

Figure 17 A) The expression of *Cyp2b10* on PLGA vs PLGA with squalene. *, P value of 0.0238
B) The expression of *Cyp2b10* on glycine vs glycine with squalene. *, P value of 0.0172

5- Discussion

In this study, there were four objectives, the characterization of the DNA element responsive to squalene in the murine gene *Cyp2b10*, to compare different carriers to increase the squalene delivery efficiency, to study the effect of squalene in comparison to lanosterol in *Cyp2b10* promoter activity and to investigate the expression level of *Cyp2b10* in presence of squalene. This study has found that the 350 bp fragment has a higher promoter activity than the full promoter, that the PLGA is the best carrier for squalene, and that it causes a reduction in expression of *Cyp2b10* but cannot give a clear answer to effects of lanosterol compared to squalene.

Our results indicate that the small fragment of 350 bp of the *Cyp2b10* promoter that contains some of the important motifs, does indeed possess a greater activity of luciferase referred to alkaline phosphatase than the full promoter, with an increase in activity of 41% as seen in figure 10, with similar results on across the different experiments with just a few cases where it didn't present an increase but could be attributed to manual mistakes while performing the experiments.
In terms of its response to squalene the small 350 bp fragment also presents an increase in activity against the full promoter, but with no significant statistical

difference, this increase while not of statistical significance has been present across the different carriers and concentrations, thus this increase in activity could be referred to the natural increase of promoter activity rather than the effect of squalene.

In terms of the best carrier for the squalene was PLGA with the 30 µM causing a 60% decrease in activity, while the worst carrier was ethanol with just a 12% decrease in activity, and gelicelina being slightly above that of squalene. At 50 µM of PLGA there is also a significant decrease in activity as seen in figure 12. At smaller concentrations some experiments show a decrease while in others it does not, so it can't be confirmed a decrease at 15 µM and 7.5 µM of PLGA, this can be attribute to lower sensitivity at these concentrations. For ethanol and gelicelina as their differences in the decrease are smaller than in PLGA making it difficult to assess the changes at difference concentrations due to the discrepancies in-between experiments. But for gelicelina a test with higher concentrations of squalene with gelicelina, 100 µM as shown in figure 15, were made that there was a significant reduction. Thus, with the results of the different experiments conducted for gelicelina it is possible that at lower gelicelina concentrations as a carrier is not effective enough, while at higher concentrations it performs better, or that the increase in squalene overcomes the lack of efficiency of gelicelina as a carrier, more studies would be necessary in order to provide a clear answer.

As for the use of lanosterol to compare the promoter activity in cholesterol synthesis, there were several problems during the experiments, with contamination and failure to successfully transfect cells. This means there is only one set of data to draw conclusion from instead of several sets of data like for the carriers for squalene. Therefore, to extract a definitive result is not possible, as more experiments would be needed to make sure that the results obtained are not due to chance, a human error or the used vehicle.
And lastly in regard to the expression levels in the presence of squalene, an RT-qPCR was done with different conditions, where it was shown that squalene with PLGA and gelicelina caused a decrease of *Cyp2b10* expression.

These findings that squalene reduces the expression of *Cyp2b10*, is unexpected compared to previous studies [12], where the expression of this gene was increased in *Apoe*-deficient mice receiving a different diet with squalene. This discrepancy could be attributed to the different cell lines used, in previous study used HepG2 cell lines, while in this study the AML12 cell line was used. If the cell lines were not the main factor for this difference in results, maybe these new carriers (PLGA and gelicelina) were the cause for this discrepancy as the previous study did not use them.

With squalene and its function of alcohol dehydrogenase activity in the metabolism of ethanol [40]. The oxidation that the ethanol suffers generates ROS with the capacity to cause liver damage, and with enough damage to liver disease [55]. So, with the reduction of its expression it could lead to less ROS generation leading to less chances to generate liver damages helping prevent liver disease, while buffered with the squalene antioxidant nature to repair oxidative damage [61].

In other studies, other groups have used different components that affect the expression of *Cyp2b10*, such as lipopolysaccharides [62] where the expression level of multiple CYPs, such as *Cyp3a11*, *Cyp2c29* and *Cyp2c55* was reduced, but *Cyp2b10* was not affected. Another study [63], that used oleanolic acid found that this component increase the expression of *Cyp3a11* and reduced the expression of *Cyp2b10*, *Cyp1a2* and *Cyp4a10*. In a different study [64], the use of perfluoro carboxylic acids increased the mRNA expression of *Cyp2b10* 20 fold, while *Cyp3a11* was 2 fold and *Cyp4a14* was 32 fold. So, while in this study is focused on squalene and the three different carriers there is still more substances that influence *Cyp2b10* that can be used to characterize its expression.

6-Conclusions

In conclusion, the *Cyp2b10* 350 bp segment presents a higher promoter activity than the full promoter, PLGA is a better carrier than gelicelina and ethanol and that the expression of *Cyp2b10* is reduced by the presence of squalene. But no clear answer can be given for lanosterol compared with squalene in the promoter activity.

6-Conclusiones

En conclusión, el segmento *Cyp2b10* de 350 pb presenta una mayor actividad promotora que el promotor completo, el PLGA es mejor portador que la gelicelina y el etanol y que la expresión de *Cyp2b10* se ve reducida por la presencia de escualeno. Pero no se puede dar una respuesta clara para el lanosterol en comparación con el escualeno en la actividad del promotor.

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