Tampere University of Applied Sciences

Functionality of the *in Vitro* Hepatic Models – Comparison of Primary Cells and Hepatocarcinoma Cell Line

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ABSTRACT

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Functionality of the *in Vitro* Hepatic Models – Comparison of Primary Cells and Hepatocarcinoma Cell Line

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This thesis was conducted with Finnish Hub for Development and Validation of Integrated Approaches (FHAIVE). FHAIVE research group is a part of Faculty of Medicine and Health Technology of Tampere University. The group focuses on replacing animal testing with *in vitro* models, computing and machine learning.

The EU funded project LiverTarget aims to invent efficient medical treatments to cardiometabolic diseases which are one of the leading causes of death in the world. LiverTarget aims to use RNA inhibition to reduce the expression of inflammatory genes which could work as a treatment to these diseases. The aim of the thesis was to define an experimental setup for the research group's LiverTarget project. The objective of the thesis was to culture and compare primary hepatocytes and hepatocarcinoma cell line and their metabolic activity. This was done by studying their metabolic activity with commercial kits that measure secretion of urea and albumin and by measuring expression of genes typical to liver and important to metabolism with reverse transcriptase quantitative polymerase chain reaction.

The results show that the primary cells maintain their liver-like functions better than the hepatocarcinoma cell line. Urea production was higher in the primary cells. Also, the gene that encodes the production of albumin, *ALB*, and urea linked gene *ARG1* were more expressed in the primary cells which indicates towards higher secretion. The gene expression was higher in the primary cells in most of all the other tested genes as well.

The results of this thesis will be used in the implementation of the LiverTarget experiments. The test setup was mostly applicable but will need some improvements before the actual project experiments begin. In the future, repetitions of the test are needed and a new cell line with characteristics between the primary cells and hepatocarcinoma cells could be added to the comparison.

Key words: liver, primary hepatocyte, hepatocarcinoma cell line, metabolism, gene expression

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1 INTRODUCTION

Cardiometabolic diseases are of the leading causes of death in the world. These diseases include diabetes, atherosclerotic cardiovascular disease and non-alcoholic fatty liver disease. While these diseases are highly preventable by a healthy lifestyle, medical treatments are needed for treatment and prevention of these diseases. Lifestyle changes might not be enough to reverse the damage if the disease has already progressed. (Choi et al. 2021)

The EU funded project LiverTarget aims to come up with efficient medical treatments for these diseases. Specific ceramide lipids are proven to be factors behind the diseases. Elevated levels of ceramide lipids are linked to for example obesity which is one factor of cardiometabolic diseases. Ceramides are produced in many parts of the body, but the synthesis is especially high in the liver. Therefore, LiverTarget aims to come up with a treatment to target these synthesis pathways in the liver with RNA inhibition to reduce the production and possible inflammation. (European Commission 2022)

This bachelor's thesis was done for FHAIVE (Finnish Hub for Development and Validation of Integrated Approaches). FHAIVE research group is a part of Faculty of Medicine and Health Technology of Tampere University. The group focuses on replacing animal testing with *in vitro* models, computing and machine learning.

The aim of the thesis is to define an experimental setup for the research group's LiverTarget project. Results of the thesis can be used in the LiverTarget project's experiments.

The objective of the thesis is to culture and compare primary hepatocytes and hepatocarcinoma cell line and their metabolic activity. Their metabolic functions and gene expression are compared to find the most efficient way to implement the LiverTarget project experiments. First research task is to culture the cells for the study. Second task is to study metabolic activity with commercial kits that measure secretion of urea and albumin. Third task is to use RT-qPCR to measure expression of genes important to metabolism.

First research task will provide information on how the cells will survive and grow in the designed setup. Second research task will determine if the hepatocytes maintain their life-like functions *in vitro*. Third task will provide information on which genes are expressed *in vitro* conditions. Genes chosen for this thesis are targets in the LiverTarget project plan and important in the context of cardiometabolic disease treatment. In this setup, primary hepatic cells and hepatocarcinoma cells are cultured and compared to determine the best cells to use in further research.

2 FUNCTIONS OF THE LIVER

Liver is the largest gland of the human body. In an average adult it weighs approximately 1,4 kg. It is composed of multiple components. (Tortora & Derrickson 2009, 945) Liver is a vital part in many important metabolic functions such as carbohydrate, lipide and protein metabolism. In these metabolic reactions liver synthesizes for example glucose, proteins such as albumin and fibrinogen, and lipoproteins such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Liver also breaks down different toxins and waste products such as bilirubin, ammonium, hormones and drugs, then proceeds to excrete them in urea or bile. (Mitra & Metcalf 2012; Dutta et al. 2021)

Hepatocytes are parenchymal cells of the liver, meaning that they take up most of the tissue of the organ and perform the important functions. They take approximately 80 % of liver's total mass. The rest of the cells are non-parenchymal cells that take substances such as proteins, toxins, hormones and nutrients from the blood plasma into the liver for the hepatocytes to break down. These non-parenchymal cells in the liver are for example endothelial cells of the hepatic sinusoids, stellate cells, leukocytes and Kupffer cells that function as macrophages. (Godoy et al. 2013)

2.1 Hepatocytes

Hepatocytes are highly functional and specialized endothelial cells in the liver. They perform several metabolic, endocrine and secretory functions. Hepatocytes perform most of liver's functions listed above. Together hepatocytes form a complex called hepatic lamina. The lamina consists of hepatocytes, bile canaliculi and hepatic sinusoids. (Tortora & Derrickson 2009, 945-946)

Hepatocytes have a polyhedral structure. They have a round nucleus in the center of the cell. Some hepatocytes have two nuclei, containing twice as much chro-

mosomes as a normal nucleus has, meaning they are often tetraploid. The number of cells that have two nuclei increases during injuries or regeneration. (Dutta et al. 2021)

Hepatocytes have a big role in detoxifying of the blood. One important function of hepatocytes is drug metabolism, meaning they break down for example aspirin, paracetamol, salicylates and antihistamines. Drug metabolism in hepatocytes is mostly controlled by cytochrome *P450* enzyme superfamily genes. The superfamily contains approximately 154 genes. All the genes are named in a particular way, for example Cytochrome Family 2 Subfamily C member 8 (*CYP2C8*). (Dutta et al. 2021; GeneCards 2023d)

2.1.1 Plasma protein albumin

Albumin is a plasma protein synthesized by hepatocytes in the liver. Small proportion of synthesized albumin is stored in the liver, but most is excreted into the bloodstream. In healthy humans, half of plasma protein content is albumin. Albumin's functions are to act as a modulator of oncotic pressure of blood vessels' plasma and to transport small ligands in plasma. (Moman, Gupta & Varacallo 2022)

Albumin is a simple, single chain polypeptide. The single chain consists of 585 amino acids. Albumin's tertiary structure consists of three domains, that fold independently. Each domain has two subdomains. These domains form a shape that could be described as heart-like. The domains are connected by disulfide bridges. (Raoufinia et al. 2016) The structure contains multiple binding sites which enables the protein to act as a carrier to many substances. It also has a free cysteine position which allows the protein to be a part of reactions such as oxidation, thiolation and nitrosylation. Despite all above, the structure of albumin is highly stable. (Spinella, Sawhney & Jalan 2016)

The gene encoding the production of albumin is called *ALB* (GeneCards 2023a). Any mutations in this gene will lead to an abnormal protein. The synthesis of albumin takes place in the liver and is performed by hepatocytes. Normal albumin

concentration in healthy adults is 3.5-5 g/dl and approximately 12-25 grams of albumin is synthesized per day. (Raoufinia et al. 2016)

Various factors affect the albumin synthesis rate. Albumin synthesis only works routinely when the body is nourished properly. For example, poor nutritional state, such as fasting, inhibits the synthesis. Conversely during illness or growth, the liver may increase the synthesis to meet the increased need of protein. (Moman et al. 2022)

Possibly the most recognized function of albumin is to control the oncotic pressure of plasma. This means that albumin helps to maintain a proper balance of fluids between the blood vessels and tissues. Other important functions are to bind and transport different substances. Albumin can bind molecules in various ways. Different subdomains of the structure have different affinity towards different molecules. This way albumin can bind metal cations, for example nickel, cobalt and copper. Albumin has a negative charge which allows it to bind positively charged molecules. (Spinella et al. 2016)

Albumin transports several substances by reversely binding to them. Albumin acts as a receptor to these ligands and transports them. Endogenous molecules binding to albumin are for example bilirubin, fatty acids, steroids and vitamin-D. Exogenous molecules are for example various drugs, ibuprofen and some penicillins. (Spinella et al. 2016)

2.1.2 Secretion of urea

Urea is one of the human body's ways to get toxins and waste products out of the body. In the liver, hepatocytes break down substances such as nitrogen or ammonia and convert them into urea. Urea then enters the kidneys via bloodstream and gets converted into urine and excreted out of the human body. (Yokoyama 2021)

Urea is the main way of excreting excess nitrogen. Nitrogen is usually in the form of ammonia that forms for example in protein catabolism. Ammonia is broken

down in a reaction series called urea cycle. Ammonia can accumulate in the body if the process is not working efficiently. Urea cycle is performed by the hepatocytes of the liver. The reaction starts in the hepatocytes' mitochondria and then continues in their cytoplasm. (Barmore, Azad & Stone 2022; Campbell, Farrel & McDougal 2016, 714-715)

Ammonia and carbon dioxide get converted into carbamoyl phosphate in a hydrolysis reaction in the hepatocyte's mitochondria. Carbamoyl phosphate then enters the urea cycle that is presented in figure 1. Figure does not include enzymes and required ATP-molecules. Conversion of carbamoyl-phosphate to citrulline takes place in the mitochondria. All the reactions after that take place in the hepatocytes' cytosol. (Campbell et al. 2016, 716-717; Yokoyama 2021)

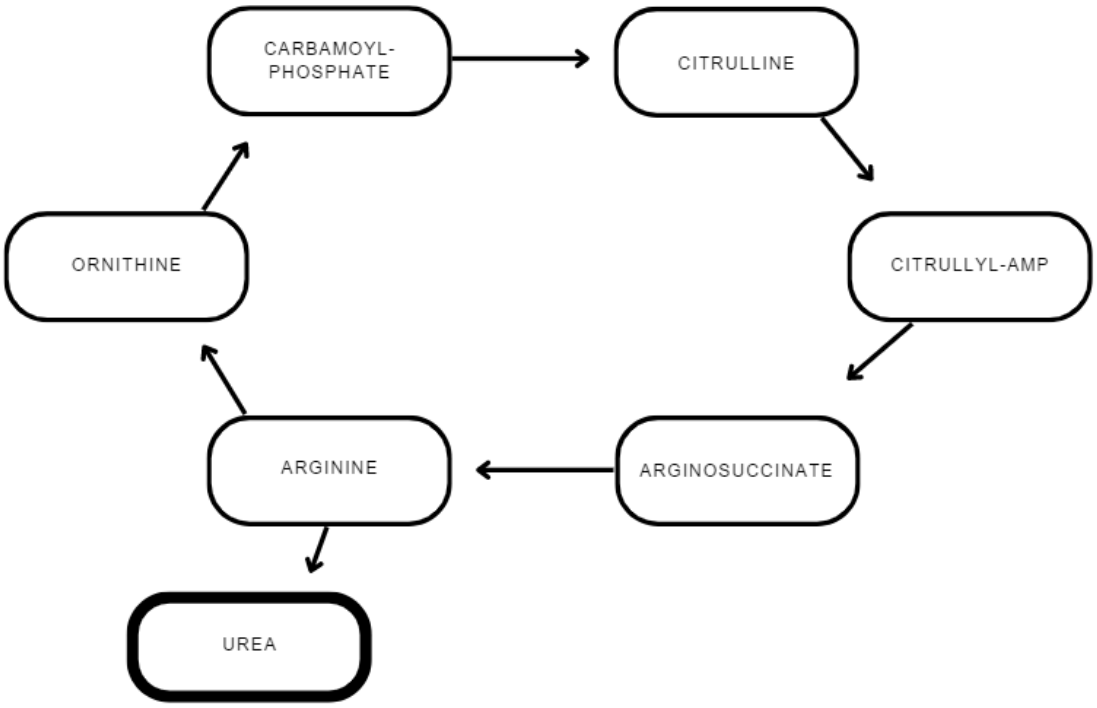

FIGURE 1. Urea cycle, simplified (Campbell et al. 2016, 717)

Last step of the urea cycle is the formation of urea and ornithine from arginine. In the hydrolysis reaction, H_2O enters the cycle and breaks down arginine to ornithine and urea as shown in figure 2. Ornithine gets transported back to the mitochondria and is a part of the next urea cycle. Urea is transported to the kidneys via the bloodstream. (Campbell et al. 2016, 716-717)

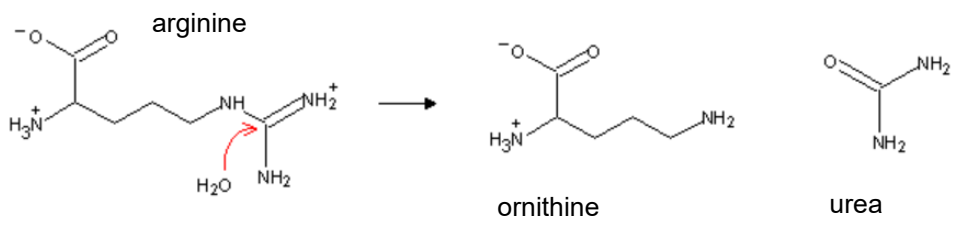

FIGURE 2. Arginine conversion to urea, modified (Silva n.d.)

The reaction of figure 2 is catalyzed by enzyme arginase. Arginase is a protein coded by a gene called arginase 1 (*ARG1*). By catalyzing this reaction, the protein and therefore the gene *ARG1* controls the production of urea. (GeneCards 2023b)

2.2 Liver's connection to cardiometabolic diseases

Cardiometabolic diseases are for example type 2 diabetes, atherosclerotic cardiovascular disease and non-alcoholic fatty liver disease. Ceramides and their synthesis are proven to be linked to cardiometabolic diseases. The synthesis is especially high in the liver. Ceramides are a group of lipid-based substances that are also precursors to sphingolipids. Sphingolipids are functional parts of lipid bilayer structures. Ceramides can accumulate to different tissues such as blood vessels, heart and liver. The accumulation is also closely linked to obesity. (Choi et al. 2021)

Sphingolipid synthesis is a complex pathway that starts from palmitoyl-CoA and serine. The pathway is controlled by many genes, for example Serine Palmitoyltransferase Long Chain Base Subunit 3 (*SPTLC3*) and Ceramide Synthase (CERS6). They control the part of the pathway from palmitoyl-CoA and serine to ceramides. From ceramides to sphingomyelin, pathway is controlled by sphingomyelinases, for example Sphingomyelin Phosphodiesterase 1 (*SMPD1*). After this part, sphingomyelin is hydrolyzed to sphingolipids. (Choi et al. 2021; GeneCards 2023c, g, h)

Other associated factors of non-alcoholic fatty liver disease and obesity are increased levels of low-density lipoproteins (LDL) and decreased levels of high-density lipoproteins (HDL). LDL circulates in blood and since blood flows through the liver, LDLs can bind to LDL receptors in the liver. Myosin Regulatory Light Chain Interacting Protein (*MYLIP*) is a gene that degrades said LDL receptors. Therefore, it can inhibit LDL intake to the liver. (Tacer & Rozman 2011; GeneCards 2023f)

One factor linked to obesity is increased levels of cortisol in the body. Cortisol is a hormone synthesized for example in the liver from cortisone. This reaction is regulated by the expression of gene Hydroxysteroid 11-Beta Dehydrogenase 1 (*HSD11B1*). Increased expression of *HSD11B1* has been observed in humans with obesity. Obesity is a big risk factor for cardiometabolic diseases. (Brix et al. 2021; GeneCards 2023e)

3 METHODS FOR STUDYING THE LIVER

As explained before, the liver has various metabolic functions. This makes liver cells, especially hepatocytes, a great model for toxicity testing and chemical safety evaluation. Their potential has been shown in many studies and publications in the past decades. There are multiple ways of characterizing the *in vitro* hepatic models to determine the optimal model for a specific study. (Vinken & Hengstler 2018)

Few factors can be studied when characterizing the *in vitro* models. These include for example morphology, viability, functionality and toxicity. Morphology can be studied with a microscope. Normal hepatocytes have a polygonal form, but a shrunken or swollen look of the cells is an indication of cell death. Viability is an important marker to study. A peak of cell death can be detected in the beginning of the culturing, since not all the cells will adjust to the artificial conditions. Viability can be assessed when counting the cells. (Vinken & Hengstler 2018)

Since the hepatocytes perform multiple metabolic functions, it is important to test the functionality of the *in vitro* model. This is done by studying the functionality itself and by studying the regulation of the metabolic functions via gene expression. Functionality itself is determined by studying if the hepatocytes have produced liver specific products such as urea or albumin. Gene expression can be studied in several ways, one example is RT-qPCR. Breaking down toxins is one important function of the hepatocytes. Therefore, exposing the hepatocytes to different toxins and determining if they can break down the toxins is a great indicator of hepatocyte functions. (Vinken & Hengstler 2018)

Primary cells or cell lines from cancerous cells are often used as *in vitro* models of the liver. Primary cells are isolated directly from tissues or organs. Primary cells maintain their functions and phenotypes even *in vitro* after isolation. For example, primary hepatocytes maintain most of their metabolic functions. However primary cells have a limited life span, availability and differences caused by different donors. After isolation, primary cells lose their life like functions quite rapidly which decreases their usefulness in studies. (Sjogren et al. 2014; Gerets et al. 2012)

Hepatoma cell lines are another option for primary cells. Human hepatoma cell lines are commonly used in studies for their high availability, nearly unlimited lifespan, easy handling and stableness. However, the cell lines' metabolic functions are limited, and the expression of certain genes is lower. Most widely used hepatocarcinoma cell line is HepG2. The cell line was isolated from a differentiated hepatocellular carcinoma of a 15-year-old Caucasian male. (Donato, Tolosa & Gómez-Lechón 2014; Kang et al. 2016)

3.1 Studying of the functionality

Albumin and urea production are markers of hepatocyte functionality. Their production can be studied for example with commercial kits. Commercial kits often measure the produced amount of a substance from the growth medium. (BioAssay Systems 2017)

BioAssay Systems QuantiChrom™ Urea Assay kit is a quantitative colorimetric kit to determine urea from samples. The kit measures urea directly from the samples without pretreatment. The analysis is performed on a 96-well plate. Chromogenic reagent is added to the samples. It forms a colored complex with the urea of the samples. The intensity of the color can be measured and is directly proportional to the sample's urea concentration. Intensity can be measured with a microplate reader. One standard is added to the well plate. Sample concentrations are calculated using the standard concentration and the kit's own formula (formula 1). In the formula, *OD* means optical density, *n* dilution factor and *STD* standard. (BioAssay Systems 2017)

$$\text{urea concentration} = \frac{\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{blank}}}{\mathrm{OD}_{\mathrm{standard}} - \mathrm{OD}_{\mathrm{blank}}} \cdot \mathrm{n} \cdot \text{STD concentration} \qquad (1)$$

Enzyme-Linked Immunosorbent Assay (ELISA) detects desired compound from a sample. Human albumin sandwich ELISA kit detects the amount of protein albumin in a sample, for example cell culture media. The protocol is performed on a 96-well plate that is coated with an antibody. The plate is washed between every step. First, samples are pipetted to the wells where their antigen binds to

the antibody of the wells. Then biotin conjugated secondary antibody is added to the wells. The secondary antibody binds to the antigens of the samples. Then streptavidin with horseradish peroxidase (HRP) is added and binds to the secondary antibody. Finally, tetramethylbenzidine substrate is added and due to enzymatic degradation, HRP forms an absorbance detectable form. Absorbance can be measured with a microplate reader. (Invitrogen 2021)

Microplate readers are designed to measure different reactions or analytes from a well of a well plate. It is an optical system that detects optical signals from biochemical processes. The reader can detect specific wavelengths coming through the samples. Many devices can measure multiple things such as luminescence, fluorescence, absorbance and optical density. (BMG Technology n.d.)

3.2 Studying of the gene expression with reverse transcriptase quantitative polymerase chain reaction

Polymerase chain reaction (PCR) makes it possible to amplify even small amounts of DNA. The reaction copies both strands of the complementary DNA of interest. Since the reaction involves high temperatures that would normally denature the DNA polymerase, an enzyme extracted from deep-sea bacteria is used instead. Their Taq DNA polymerase tolerates the high temperatures and maintains its functions. (Campbell et al. 2016, 401-402)

During the PCR the samples are first heated to 90ºC to separate the DNA strands. Second part of the process is to cool down the samples so that the primers added can anneal themselves to the DNA strands. The temperature is raised again to allow the Taq polymerase to synthesize the new DNA. This reaction doubles the amount of DNA. The automated process of separating the DNA strands, annealing the primers and synthesizing the new DNA strands is repeated until desired amount of DNA is produced. (Campbell et al. 2016, 402)

Quantitative polymerase chain reaction is an advanced version of the PCR. qPCR allows a quantitative analysis of the DNA during the process. This is done by adding a fluorescent dye to the sample and using a fluorometer during the PCR

process. qPCR determinates the amount of the DNA in the original sample. (Campbell et al. 2016, 402-404)

Fluorescent dye is added to the samples in qPCR process. Intercalating fluorescent dyes bind to double-stranded DNA molecules. It is not target specific and will bind to any PCR product but when primers are designed correctly it should bind to the desired DNA molecules. The dye intercalates to the DNA molecules during the extension phase of the reaction. After binding, the dye starts to fluorescence which can be measured with a fluorometer. (Butler 2012, 55; Mészáros 2022)

EvaGreen® is a fluorescent nucleic acid dye similar to more known dye SYBR® Green. Like any fluorescent dye, EvaGreen binds nonspecifically to all DNA molecules which is why specific amplification is highly important. EvaGreen has low PCR inhibition and special Sso7d technology. Sso7d is a double-stranded DNA binding protein that stabilizes the DNA polymerase. EvaGreen creates a more robust PCR signal than SYBR green since it can be used in higher concentrations because of its low PCR inhibition. (Biotium 2019; BioRad n.d.)

Reverse transcriptase qPCR is used when measuring the gene expression. Relative RT-qPCR is used when different sample gene expressions are compared. In this method, RNA is extracted from the samples. Before the RT-qPCR analysis, the samples must be reverse transcribed to complementary DNA. This is done by using reverse transcriptase enzyme. cDNA samples are then analyzed with qPCR. (Neidler 2017)

Reference genes, in other words known as housekeeping genes, are used in RT-qPCR for normalization. Using the housekeeping genes is the most common way of normalizing the RT-qPCR data. Housekeeping genes are stable, expressed and essential in the tissues of interest and do not change during the experiment. Use of a single housekeeping gene is not enough for normalization and the number of housekeeping genes must be determined for each experiment individually. (Bustin et. al. 2009)

Quantification cycle (C_q) is the cycle in qPCR where the fluorescence value raises above the threshold value and starts to show on the measurements. Threshold cycle (C_T) is another used name for the same cycle. (Bustin et. al. 2009) Usually a cutoff C_q value is determined for the study. Cutoff value is determined for each study separately and is often based on reaction end-cycle, meaning that the reaction has already come to its end. A sample with higher value than the cutoff would be classified as an outlier and removed from the calculations of the results. Often this higher value is thought to be caused by nonspecific amplification or contamination. (Caraguel et al. 2011)

Common way of analyzing qPCR results is the $\Delta\Delta C_q$ -method (Bustin et. al. 2009). In $\Delta\Delta C_q$ -method, first the housekeeping gene C_q value is subtracted from the sample C_q values, this creates the ΔC_q-value. One sample group is then chosen as the control group. An average is determined from the ΔC_q values of the control group. This average is then subtracted from the ΔC_q-values of all the samples, creating the $\Delta\Delta C_q$-value. Finally, a value of $2^{-\Delta\Delta Cq}$ is calculated. Sample results can then be compared to understand which genes are more expressed in the different samples. (Top Tip Bio 2018)

4 EXPERIMENTAL PART

4.1 Cell culture

4.1.1 Culturing of HepG2 cells

HepG2 cells were originally from ATCC, for this study they were received from another research group. The cells had been cultured before and had a passage number of 4. HepG2 medium was prepared following the standard operation procedure 82 (FHAIVE 2016a). Sigma-Aldrich Minimum Essential Medium Eagle and heat inactivated Gibco Fetal Bovine Serum (FBS) were used to make a 10 % FBS medium.

Cells were thawed following the standard operating procedure 83 (FHAIVE 2016b). After thawing, the cells were counted using Sigma TrypanBlue™ and Cellometer Auto T4 Plus device. A 2-fold dilution was made with TrypanBlue and cell suspension.

There were $6{,}96 \cdot 10^6$ cells counted to be in 1 ml of the suspension. Cell suspension volume was adjusted to density of 70 000 cells/ml. Cells were plated to a sterile non-treated 12-well plate with 1 ml of cell suspension in each well. Cells were plated to two well plates, one for samples and one for growth curve. Only the two middle lines of wells were used from each well plates. The cells were incubated in 37ºC.

The rest of the cells were plated to two cell culture flasks to grow for future well plate plating. For the cell culture flasks, the cell suspension volume was adjusted to 500 000 cells/flask.

Cells were counted from the growth curve plate on days 1, 4 and 5. Each time, the cells were counted from two wells. The adherent cells were detached from the wells with Gibco Tryple™ Express. Cells were again counted with Sigma TrypanBlue™.

Cells from the two flasks were later plated on two 12-well plates. Volume was adjusted to 140 000 cells/ml. Cells were counted on days 3 and 5. Only one well was counted each time.

4.1.2 Culturing of LiverPool cells

Before plating, two 12-well plates were coated with collagen. A 50 µg/ml solution of collagen was prepared. The solution was pipetted to the well plate at volume of 5 µg/cm^2. The well plates were incubated in 37ºC overnight. The well plates were washed with phosphate-buffered saline (PBS) before use.

LiverPool® 10-donor mixed gender human hepatocytes from Bioivt were used in this study. For thawing and plating LiverPool cells, commercial InVitroGrow CP medium was used. Thawing of the cells was performed with the manufacturer's instructions (Bioivt 2018). Cells were counted with TrypanBlue. 10,825 · 10^6 cells were calculated to be in cell suspension. Cell suspension volume was adjusted to 700 000 cells/ml. The cells were plated to the collagen coated 12-well plates.

Commercial InVitroGrow HI medium was changed to the cells on day 1. InVitroGrow HI medium was used from day 1 on. Cells were counted for the growth curve on days 2 and 5.

4.1.3 Sample collection

Sample collection procedure was the same for both cell lines. Samples were collected on day 5. 400 µl medium from the wells was collected in separate Eppendorf tubes for urea measurements. Another 400 µl of medium was collected to separate Eppendorf tubes for albumin measurements.

RNA extraction was performed with QIAGEN RNeasy Mini Kit and started with collecting the cells from the bottom of the well plate. The kit had its own protocol, RNeasy Mini Handbook which was followed (QIAGEN 2019). Most of the reagents came with the kit. The wells were first washed with PBS and then the cells

were lysed to a buffer solution. All cell lysis samples were kept in -80 °C freezer until the next step of the analysis.

4.2 Urea and albumin kits

Urea kit was performed with the kit's own instructions on a 96-well plate. The kit in use was QuantiChrom™ Urea Assay Kit (DIUR-500). (BioAssay Systems 2017). Urea samples were not diluted and the protocol for low urea samples was followed. That protocol included the use of one standard with concentration of 5 mg/dl. Both growth mediums, HepG2 medium and Invitrogrow HI medium were run as blanks for the samples. Samples were incubated for 50 minutes. Optical density was measured with Tecan Spark Multimode Plate Reader at 430 nm.

Albumin kit was performed with the kit's own instructions (Invitrogen 2021). Invitrogen Human Albumin (ALB) ELISA kit was used. 100- and 1000-fold dilutions were made from the albumin samples. Both growth mediums, HepG2 medium and Invitrogrow HI medium were run as blanks for the samples. A longer protocol was used, meaning that the well plate was incubated overnight at 4ºC with gentle shaking after standard and sample application. Rest of the analysis was performed on the following day. Absorbance was measured at 450 nm with Tecan Spark Multimode Plate Reader.

4.3 RT-qPCR analysis

The steps of the RT-qPCR analysis are presented in figure 3.

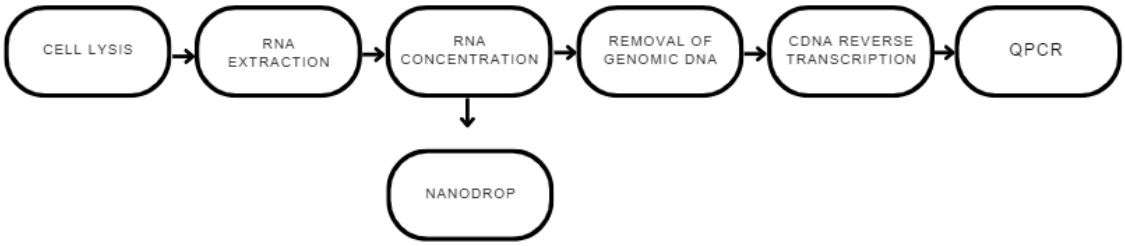

Figure 3. RT-qPCR analysis

RNA extraction was continued from the cell lysis with the QIAGEN RNeasy Mini Kit instructions (QIAGEN 2019). The kit included its own special spin columns. First, ethanol was added to the cell lysis samples to homogenize the lysate. The ethanol and sample solution were added to the spin column. The goal of this step was to bind the RNA to the spin column. After this, the RNA was washed with wash buffers three times. Last step was to elute the RNA sample with RNAse-free water to the collection tube. Some of this eluted sample was taken to another collection tube for analysis of concentration and purity. The samples were stored in a -80 °C freezer.

NanoDrop RNA concentration and purity analysis was performed with Thermo-Scientific NanoDrop 2000 spectrophotometer. 2 µl of the samples was pipetted onto the device pedestal. Absorbance was measured with wavelengths of 230 nm, 260 nm and 280 nm. This way ratios of A260/280 and A260/230 could be calculated to estimate the purity of the samples. Device also provided results for sample concentrations measured in 260 nm wavelength. NanoDrop results (Appendix 1) were used to calculate dilutions of the samples for the next steps. Two of the samples were left out of the next steps for their significantly lower nucleic acid concentrations or A260/230 value.

Next step of the analysis was removal of genomic DNA from the RNA samples. This step was performed with instructions from ThermoScientific for DNase I, RNase-free product kit (ThermoFisher 2016). 10X reaction buffer with $MgCl_2$ and RNAse free water instead of DEPC-free water were used. For this step, HepG2 samples were diluted to 30 ng/µl of RNA and LiverPool samples to 50 ng/µl of RNA. Calculations were based on the NanoDrop results (Appendix 1). Two different master mixes were prepared for the different cell types with kit instructions. Final concentration of the samples was 500 ng of RNA per sample. Samples were stored in a -80 °C freezer until the next step of the analysis.

Next step of the analysis was complementary DNA reverse transcription. This step was performed with the instructions of Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit User Guide (ThermoFisher 2018). 2X RT master mix for 20 µl reaction was prepared with the kit instructions without the RNase

inhibitor. Reverse transcription was performed on a thermal cycler. Applied Biosystems 2720 Thermal Cycler was used with a program described in table 1. After the thermal cycle, samples were stored in -22ºC freezer until the next step.

TABLE 1. Thermal cycle

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25ºC	37ºC	85ºC	4ºC
Time	10 mins	120 mins	5 mins	hold

Final step, the qPCR analysis, was performed with BioRad SsoFast™ EvaGreen® Supermix with Low ROX. Kit's own protocol was followed (BioRad n.d.). A primer master mix for 10 µl reaction was prepared following the kit instructions.

Primers were commercial, ordered from Fluidigm where the primers were designed for each target gene. The target genes were *SMPD1*, *CERS6*, *MYLIP*, *SPTLC3*, *CYP2C8*, *ARG1*, *ALB* and *HSD11B1*. Chosen housekeeping genes were *ACTB* and *GAPDH*. Primer master mixes were prepared for each primer separately.

cDNA samples were diluted to 10 ng of cDNA per sample. Primer master mixes and samples were pipetted to a 96-qPCR well plate. qPCR analysis was performed with CFX Opus 96 Real-Time PCR System with thermal cycling protocol described in table 2.

TABLE 2. qPCR thermal cycling protocol

	cycle 40 times		temperature ramp, 0,5ºC increments	
95ºC	95ºC	60ºC	65ºC	90ºC
30 s	5 s	30 s	5 s holds	

5 RESULTS

5.1 Cell growth curves

Growth curves were determined for each well plate. Cells were counted as described previously in the experimental part and calculations were done as in SOP instructions. Results represent how many cells were per well. Day 0 represents how many cells were plated per well. In figure 4 are the growth curves for all the plates of this study.

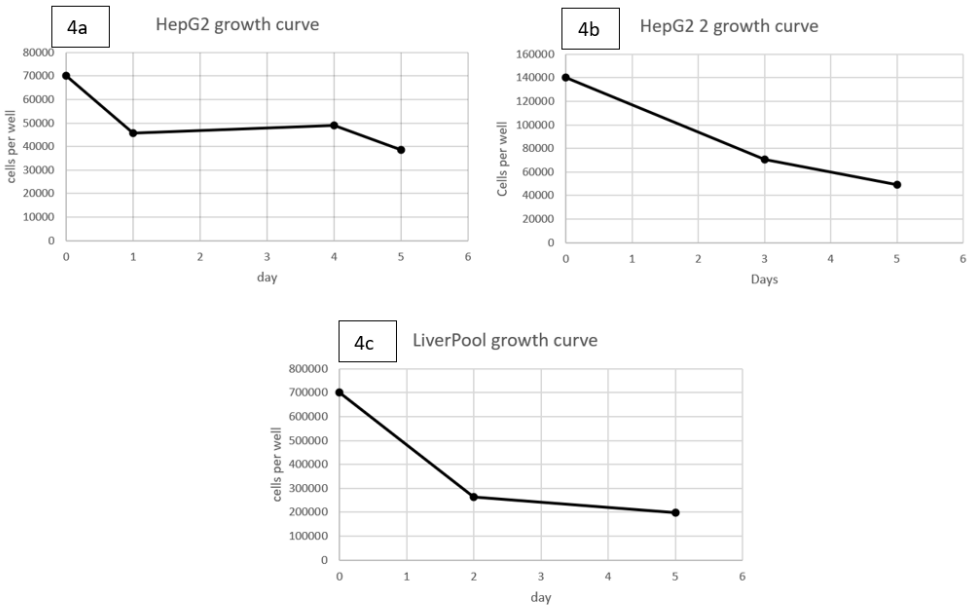

FIGURE 4. Cell growth curves for the well plates. The first plate of HepG2 cells (4a), the second plate of HepG2 cells (4b) and the plate of LiverPool cells (4c).

It can be seen from the curves, that a significant drop of cell density occurs after plating the cells. Sufficient number of cells were still left on each well plate on day 5 for the sample collection.

5.2 Urea and albumin production

Urea kit results were calculated with formula 1 that is presented in chapter 3.1. Urea kit optical density results are presented in appendix 2. Since the samples were not diluted, dilution factor *n* was 1. Water was used as a blank for the standard and growth mediums were used as blanks for the samples. Outliers were taken out from the calculations if the optical density was considerably higher than the others. After calculations, negative results were taken out. Urea secretion results in mg/dl are presented for each cell line in figure 5 as box plot with whiskers.

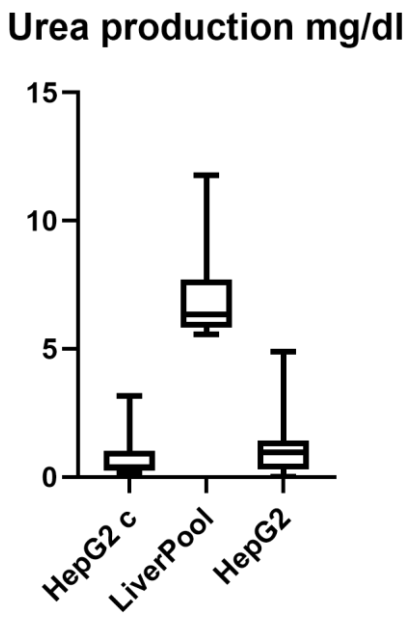

FIGURE 5. Urea production results

In figure 5 box plot, the middle line in the box shows the median of the results. Outside lines of the boxes show the upper and lower quartiles. The lines, called whiskers, show the variability outside of the quartiles. LiverPool cells have produced around 6-8 mg/dl of urea whereas HepG2 cells only around 1-2 mg/dl.

Absorbance results for albumin are presented in appendix 3. Standard curve was calculated, but since the standard results did not represent a curve and the correlation coefficient was not near 1, sample results were not calculated.

5.3 Gene expression

RT-qPCR gene expression results were calculated with delta delta C_q -method that is described in chapter 3.2. Each of the housekeeping genes were used in calculations for half of the genes of interest. *GAPDH* was used for *CYP2C8*, *SMPD1*, *SPTLC3* and *ARG1*. First well plate of HepG2 cells was used as the control group in the calculations. Original C_q values are presented in appendix 4. As explained in chapter 3.2, a cutoff value was determined for the C_q values of samples. Based on the results of non-sample controls, 35 was determined to be the cutoff value. All C_q values of target genes above that value were left out of calculations. Some outliers were taken out of the calculations if the C_q value was considerably higher than the parallel samples. Calculated results are presented as box plots with whiskers with log2 scaled y-axis. Results calculated with *GAPDH* as a housekeeping gene are presented in figure 6.

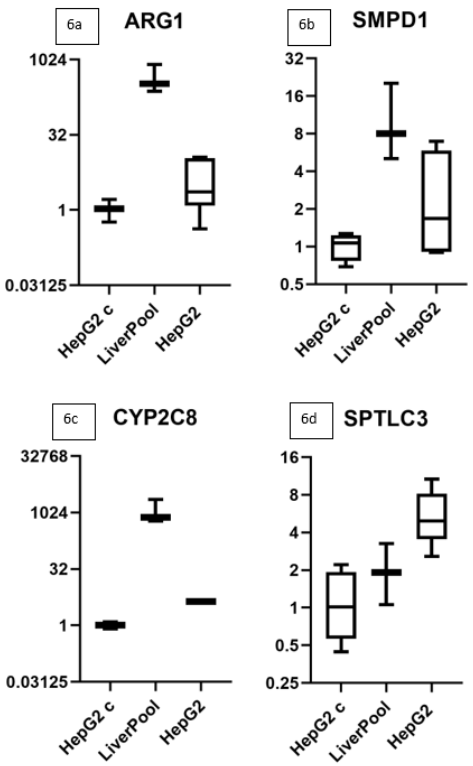

FIGURE 6. Gene expressions for *ARG1* (6a), *SMPD1* (6b), *CYP2C8* (6c), and *SPTLC3* (6d).

In figure 6 box plot, the middle line in the box shows the median of the results. Outside lines of the boxes show the upper and lower quartiles. The lines, called whiskers, show the variability outside of the quartiles.

Rest of the genes of the study were *CERS6*, *MYLIP*, *ALB* and *HSD11B1*. In the calculations, *ACTB* was used as the housekeeping gene for these genes. Results for these gene are presented in figure 7. Some of the boxes and whiskers in the figure are larger or longer, meaning there is variability between the parallel samples.

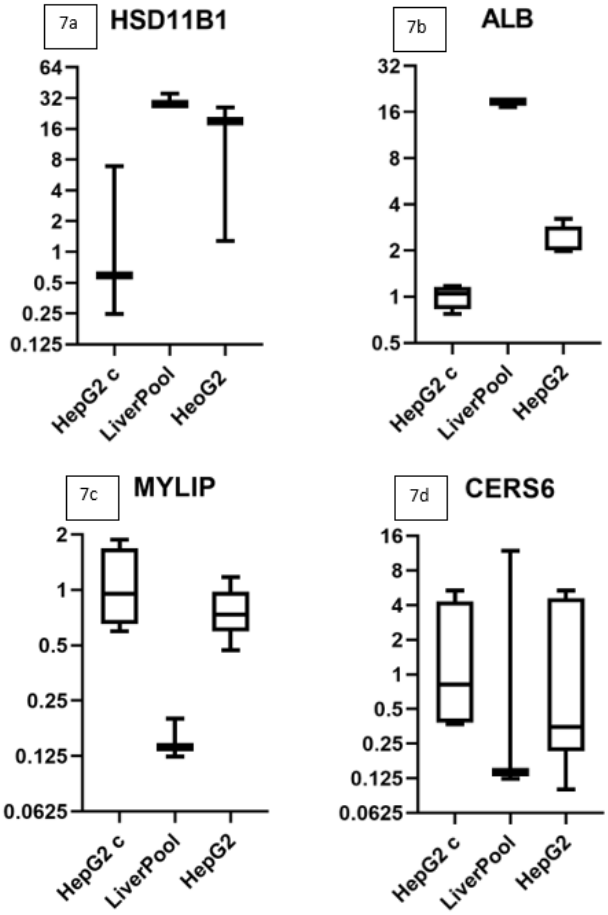

FIGURE 7. Gene expression for *HSD11B1* (7a), *ALB* (7b), *MYLIP* (7c) and *CERS6* (7d).

6 DISCUSSION

The objective of this thesis was to culture and compare primary hepatocytes and hepatocarcinoma cell line and their metabolic functions. Studying of the functionality was done with commercial kits and studying of gene expression with RT-qPCR. All the research tasks were performed. Not all the analysis produced useable results and will need new repetitions. However, enough results were gathered to successfully compare the cell lines' different functions and characteristics.

Growth curves were calculated for the cells to measure cell density and growth. A drop of cell density after plating the cells was expected as explained in chapter 3. The drop is seen in the figure 4. Hepatocytes are adherent cells and not all the cells are able to adhere to the cell culture flasks and will most likely die because of that. In addition, not all of them adjust to the artificial conditions of the *in vitro* system. (Vinken & Hengstler 2018). In the case of LiverPool cells, cells were plated in excess as instructed in the cells' user guide (Bioivt 2018). Therefore, it was also expected that not all the cells survive, since there were simply too many cells for the available medium and space on the wells.

HepG2 cells were first plated with lower cell density. There was a significant drop in the cell density as seen in figure 4a, so the second well plate was plated with higher cell density to ensure that there would be enough sample material to collect for later analysis. It can be seen from figure 4b that there has been more cell death due to the higher cell density. On day 5 both plates of figures 4a and 4b have approximately the same number of cells alive. Therefore, the higher cell density did not necessarily bring better results.

The urea kit instructions (BioAssay Systems 2017) provided the formula for calculating the results. The formula considered the standard and blanks for both the standard and the samples. The performing of the kit was successful and only a couple of the results were inconsistent and taken out. The results for each well plate are presented in figure 5. Urea production in HepG2 cells is less than 5 mg/dl and in LiverPool cells 6-8 mg/dl. In the kit instructions, predicted result for urea concentration in cell culture samples was 0,15-2,7 mg/dl. HepG2 cells seem

to mainly hit that range, but LiverPool cells are above it. In the instructions, there is no mention of what kind of cells were used, so it can be used only as directive range.

It was expected that LiverPool cells produce more urea, since primary cells maintain more tissue like functions than hepatocarcinoma cell lines. Primary cells maintain their functions for only a short period of time, but in a study this short, functions are maintained well. (Sjogren et al. 2014). Another study has been comparing urea production between primary cells and hepatocarcinoma cells and has found that primary cells produce more urea. The present study is in line with the results of that study (Kang et al. 2016).

The qPCR results support these results from the urea kit. As explained in chapter 2.1.2, *ARG1* gene codes a protein that functions as a catalyzer in the urea cycle. As seen in figure 6a, gene *ARG1* is more expressed in the LiverPool cells than the HepG2 cells. This would suggest that the urea cycle and *ARG1* are more active the LiverPool cells, therefore resulting in higher production of urea in LiverPool cells.

The results for the albumin kit could not be calculated since the standard curve points were widely spread and correlation coefficient nowhere near 1. When performing the analysis with the kit, some signs of possible failure were seen. According to the kit instructions (Invitrogen 2021), after adding the substrate, the samples in the wells would start to turn blue. The samples or standards however did not do that. After adding the stop solution, the samples were supposed to turn from blue to yellow. This did not happen either. It was assumed that not any decent results would be received from the kit.

The kit principles were followed with the exception of one misstep in dilutions of the reagents. This could have caused the negative results. Other reason could be that as explained in the chapter 3.1, the reagents have to bind to each other in all the steps of the ELISA. It is possible that already in the first step, the antigen did not bind to the antibody, and this alone would lead to a failed analysis. Multiple repetitions of this analysis have to be performed in the future.

Other study shows that albumin secretion is higher in primary hepatocytes (Kang et al. 2016). The present study also supports that with the *ALB* gene results from qPCR (figure 7b). As explained in theory chapter 2.1.1, *ALB* is the gene coding the production of the protein albumin. The gene expression is higher in LiverPool cells than HepG2 cells which means the albumin secretion would have been higher in LiverPool cells than HepG2.

It can be seen from figures 6 and 7 that most of the genes are more expressed in the LiverPool cells. It seems that only *MYLIP* (figure 7c) is clearly more expressed in the HepG2 cells. Other study shows that genes are typically more expressed in primary hepatocytes than hepatocarcinoma cell lines such as HepG2 (Gerets et al. 2012).

Biggest difference in expression between the cell lines is in the gene *CYP2C8* (figure 6c). It has been shown in other studies that HepG2 cells have significantly decreased expression of the genes of cytochrome *P450* superfamily (Kang et al. 2016; Sjogren et al. 2014; Gerets et al. 2012). As explained in chapter 2.1, *P450* enzyme superfamily is in a key position in drug metabolism in the liver. Expression of *CYP* acts as a good marker for hepatocytes' metabolic functions and how much they are maintained *in vitro*. This means, that HepG2 cells don't have enough *CYP* expression to be totally reliable and sole model for drug or toxicity studies.

Especially figure 7 shows that some boxes and whiskers are larger or longer, meaning there might be variability between the parallel samples. In *SPTLC3* gene (figure 6d), there are differences between the two HepG2 well plates which leads to the other being less expressed than the LiverPool cells and the other more expressed. It is normal for parallel samples to have variability between them. Many genes have shown to have inter-individual differences in expression. Therefore, it is possible that the genes are slightly differently expressed in different samples. (Godoy et al. 2013)

With these results, it can be concluded, as theory suggests, that LiverPool cells maintain their functions better than HepG2 cells. Functionality of the LiverPool cells was better and most of the genes were expressed more. Therefore, it would

be reasonable to continue the LiverTarget studies with the LiverPool cells. However, there are some disadvantages in using primary cell lines such as LiverPool. As explained in chapter 3, they maintain their functions much better, but are harder to acquire and handle. Main reasons hepatoma cell lines are used in many studies are their availability, steady growth and lack of donor variations. Therefore, they cannot be totally dismissed in all studies. (Godoy et al. 2013)

Another disadvantage of primary cells is their price. For that reason, an addition of a new cell line to the studies could be reasonable. Other studies show that human hepatoma HepaRG cell line would maintain its functions almost as well as primary cells (Godoy et al. 2013; Gerets et al. 2012). That would make this cell line a good candidate for continuing the studies with LiverTarget.

In the future with LiverTarget, new repetitions of these analysis must be performed to ensure reliable data. Parallel samples were already used in the present study to ensure more data and more reliable results. No direct patient samples were used in the experiments and ethical code was followed with established cell lines. The aim of this thesis was to determine a setup for LiverTarget project experiments. The results of this thesis are a good starting point for LiverTarget project. This thesis provides useful information for the research group and further studies. From this setup, new studies and repetitions can be performed.

As explained in the theory, liver's genes and functions are closely linked to cardiometabolic diseases and obesity. RNA inhibition treatment would target the synthesis pathways and therefore decrease the amount of these harmful substances. LiverTarget's RNA inhibition treatment would have a huge impact in treating these diseases.

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APPENDICES

Appendix 1. NanoDrop results

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)		A280 (Abs)		260/280	260/230	Sample Type
1	210223B1	NanoDrop	9.3.2023 10:40:57	17,4	ng/µl	0,436	0,214	2,03	0,70	RNA	40,00	
2	210223B2	NanoDrop	9.3.2023 10:44:19	35,5	ng/µl	0,886	0,448	1,98	1,21	RNA	40,00	
3	210223B3	NanoDrop	9.3.2023 10:45:38	28,0	ng/µl	0,700	0,359	1,95	0,46	RNA	40,00	
4	210223C1	NanoDrop	9.3.2023 10:46:54	48,6	ng/µl	1,216	0,644	1,89	1,00	RNA	40,00	
5	210223C2	NanoDrop	9.3.2023 10:48:10	45,7	ng/µl	1,142	0,565	2,02	1,17	RNA	40,00	
6	210223C3	NanoDrop	9.3.2023 10:49:18	30,5	ng/µl	0,763	0,389	1,96	0,26	RNA	40,00	
7	270223B1	NanoDrop	9.3.2023 10:51:11	82,9	ng/µl	2,072	1,011	2,05	0,35	RNA	40,00	
8	270223B2	NanoDrop	9.3.2023 10:52:16	53,1	ng/µl	1,326	0,635	2,09	0,09	RNA	40,00	
9	270223B3	NanoDrop	9.3.2023 10:53:18	63,9	ng/µl	1,598	0,770	2,08	0,13	RNA	40,00	
10	270223C1	NanoDrop	9.3.2023 10:54:29	76,2	ng/µl	1,905	0,948	2,01	1,64	RNA	40,00	
11	270223C2	NanoDrop	9.3.2023 10:55:39	91,5	ng/µl	2,288	1,129	2,03	0,53	RNA	40,00	
12	270223C3	NanoDrop	9.3.2023 10:56:50	102,9	ng/µl	2,571	1,257	2,05	1,33	RNA	40,00	
13	270223C3(Reblank)	NanoDrop	9.3.2023 10:58:27	102,9	ng/µl	2,573	1,276	2,02	1,33	RNA	40,00	
14	blank	NanoDrop	9.3.2023 11:00:02	0,0	ng/µl	-0,001	0,020	-0,03	-0,05	RNA	40,00	
15	blank(Reblank)	NanoDrop	9.3.2023 11:00:20	0,1	ng/µl	0,002	-0,002	-1,20	0,13	RNA	40,00	
16	010323B1	NanoDrop	9.3.2023 11:01:13	40,9	ng/µl	1,021	0,507	2,01	0,78	RNA	40,00	
17	010323B2	NanoDrop	9.3.2023 11:02:29	32,6	ng/µl	0,815	0,384	2,12	0,07	RNA	40,00	
18	010323B3	NanoDrop	9.3.2023 11:03:30	27,8	ng/µl	0,695	0,347	2,01	0,21	RNA	40,00	
19	010323C1	NanoDrop	9.3.2023 11:04:30	46,5	ng/µl	1,162	0,571	2,03	0,13	RNA	40,00	
20	010323C2	NanoDrop	9.3.2023 11:05:30	28,4	ng/µl	0,710	0,347	2,05	0,46	RNA	40,00	
21	010323C3	NanoDrop	9.3.2023 11:06:30	34,0	ng/µl	0,849	0,386	2,20	0,06	RNA	40,00	

Appendix 2. Urea kit optical density results

Name	OD430
Measurement wavelength	430 nm
Number of flashes	10
Settle time	50 ms
Part of Plate	A1-H12
Start Time	2023-03-08 14.26.42
Temperature	27,6 °C

<>	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		1,2953	1,5667	1,3583	2,9701	1,8501	1,2263	0,544				
D		1,2629	1,1529	1,2549	1,2548	1,2301	1,2882			0,5295	0,525	
E		1,9133	2,2656	2,1201	1,8518	1,7946	3,6523			1,1956	1,2008	
F		2,9602	1,8408	1,7354	1,7137	1,6794	1,7352			0,54	0,5315	
G		1,3938	1,6683	2,8228	1,3967	1,4313	2,2025			1,2974	1,814	
H		1,2576	1,4036	1,1983	1,3198	1,2597	1,1594					

Appendix 3. Albumin kit absorbance results

Mode	Absorbance		
Name	elisa_hrp		
Measurement wavelength		450	nm
Number of flashes		10	
Settle time		50	ms
Part of Plate		A1-H12	
Start Time		2023-03-14 13.20.10	
Temperature		25	°C

<>	1	2	3	4	5	6	7	8	9	10	11	12
A		0,0501	0,0494	0,0486	0,0485	0,0537	0,0518	0,051		0,0517	0,0503	
B		0,0533	0,0458	0,0569	0,0544	0,0551	0,0576	0,0525		0,0546	0,0579	
C		0,0586	0,0504	0,0503	0,0503	0,0524	0,0519			0,0521	0,0565	
D		0,052	0,0567	0,0544	0,0546	0,052	0,0502					
E		0,051	0,0527	0,0615	0,0527	0,0621	0,0805					
F		0,0523	0,0513	0,0503	0,0558	0,0527	0,0514					
G		0,0518	0,054	0,0508	0,054	0,0589	0,0604					
H		0,0511	0,0509	0,0515	0,0573	0,051	0,0942					

Appendix 4. qPCR, original C_T values

	GAPDH	ACTB	ALB	SMPD1	CERS5	MYLIP	SPTLC3	CYP2C8	ARG1	HSD11B1
HepG2 c	28,5263	24,6963	24,4471	30,2426	31,2019	31,6283	30,5135	35,4355	NaN	NaN
HepG2 c	22,0656	22,0059	23,155	30,0098	28,1122	32,0018	29,9097	36,0052	39,3549	NaN
HepG2 c	19,4772	20,2667	21,7851	27,9869	24,6397	29,3874	27,588	33,0472	30,1593	33,3861
HepG2 c	19,4057	21,121	22,0266	27,2083	23,3601	29,4635	28,5656	32,403	29,4859	29,4303
HepG2 c	21,4513	21,5731	22,5815	29,0744	27,4984	31,1218	28,2967	35,0665	33,0196	33,4462
LP	21,6925	21,6393	18,5213	27,3239	29,1155	33,7256	29,6038	25,7144	24,5701	27,9255
LP	23,9944	23,0201	19,9423	28,957	30,6956	35,284	30,2778	26,1423	25,0977	29,3355
LP	34,4399	35,779	30,8366	29,83	23,3313	37,0106	NaN	33,2435	32,2662	38,1042
LP	22,0096	21,9907	19,0291	25,6438	23,0755	33,5697	29,0598	25,7425	24,3923	27,9475
LP	29,2051	30,235	24,6672	33,3386	31,8398	37,013	35,1073	29,3189	28,163	34,0719
LP	29,6488	31,769	26,2601	33,6223	31,7284	26,2601	35,5696	31,0034	29,2177	35,2838
HepG2	23,5482	21,4354	21,5751	30,7684	27,6153	31,1608	28,1233	35,067	33,125	38,2045
HepG2	22,1048	21,2345	21,3944	30,2308	29,2134	30,93	27,9285	35,849	31,8558	37,4157
HepG2	21,0077	20,5647	20,0245	26,1759	22,8023	30,1765	26,4812	32,2129	28,277	27,4127
HepG2	20,6631	20,4993	20,31	26,3813	23,2321	29,5183	26,3484	31,8126	28,0368	26,9064
HepG2	21,0821	21,1228	21,2234	29,1856	27,3955	31,4592	27,7099	36,6042	33,0979	31,8706