The effect of LPS on intra- and extracellular Alkaline phosphatase levels and their relation to hepatic fibrosis

Abstract

To study the influence of different levels of LPS on the amounts of alkaline phosphatase and the relation of alkaline phosphatase to liver fibrosis. This is done with an alkaline phosphatase activity assay on the conversion of pNPP to pNP. A qPCR is done on a house keeping gene GAPDH to standardize the measurements and a qPCR is done on the RNA encoding for the Alkaline phosphatase and the TLR4 receptor. With an immunohistochemical experiment the increased amounts of IGF-II, collagen I, fibronectin and AP are visualized and analyzed. The activity assay showed no significant increase in Alkaline Phosphatase activity compared to the negative control. The qPCR of alkaline phosphatase and the TLR4 receptor did not give representable results. The immunohistochemical experiment showed increased levels of IGF-II, collagen I, fibronectin, and AP in specific areas of the liver tissue.

Introduction

The liver has different types of cells. The macrophages of the liver are called the Kupffer cells. A change in the function and activity of the Kupffer cells is induced by pathogens. With pathogens present the Kupffer cell will change to the M1 macrophage structure and M2 after the pathogen is neutralized. (4) The stellate cells are resident non-parenchymal liver pericytes and so are responsible for support the tissue when healthy and when diseased. When the tissue is healthy the stellate cells are involved in hepatic growth, immunity, and storage of nutrients like carbohydrates, lipids, and retinoid. When the tissue is triggered by pathogens the stellate cells are involved in the inflammatory response of the hepatic tissue. The stellate cells than transform into myofibroblasts that excrete extracellular matrix (collagen or scar tissue), which results in a fibrotic liver. (5)

Lipopolysaccharide (LPS) can be found in the outer membrane of most gram-positive bacteria. It has to fatty acid chains and a saccharide molecule which induces an immune response when registered by the immune system. LPS is called an endotoxin, because the gram-positive bacteria simply carry it on their membrane and do not secrete it (exotoxin). LPS is recognized by the body by the Toll-like 4 receptor (TLR4), which induces a cascade of reactions which is visible in *figure 1*. (1) This figure shows that the production of pro-inflammatory cytokines is stimulated via multiple paths and cause the increase of pro-inflammatory cytokines NFκB, Alkaline phosphatase and IRF3.

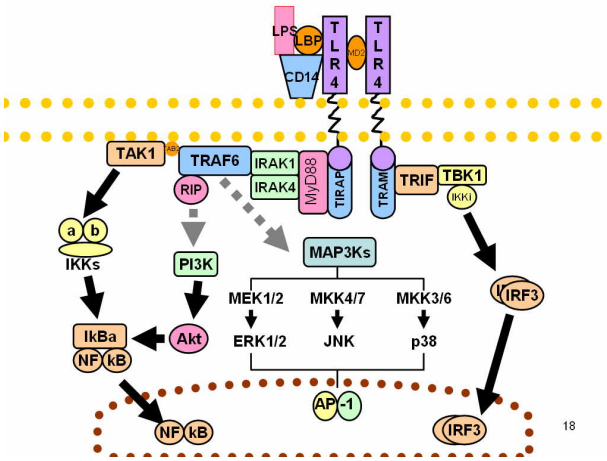

Figure 1: LPS signaling of the TLR4. (2)

The enzyme Alkaline Phosphatase (AP), when produced by the immune cells, has an anti-inflammatory effect on LPS by inactivating the molecule. This is done by dephosphorylation of the LPS molecule. (3)(11)(12)

In this paper the next question will be discussed: What is the effect of different LPS levels on the intra- and extracellular levels of Alkaline phosphatase and what is their contribution in the development of hepatic fibrosis?
Since the alkaline phosphatase production is induced by the stimulation of the TLR4 receptor, which is triggered by LPS and LPS binding protein, it is expected that the levels of alkaline phosphatase will increase with an increasing amount of added LPS. It is expected alkaline phosphatase will suppress this cascade by deactivating the inflammatory process and therefore the fibrotic process by inactivating LPS by dephosphorylation.
This is done via an alkaline phosphatase kinetic essay with the help of a substrate called pNPP which is converted to pNP. LPS will be added in different concentrations to trigger the cells in different urgencies to produce alkaline phosphatase and therefore get increasing amounts of converted pNPP at higher added levels of LPS. This elimination of a phosphate group leaves pNP with a yellow colour. During a 30 minutes assay the adsorption of the fluids with the substrate is analyzed. The mRNA levels of the AP and the TLR4 receptor are also analyzed using a qPCR with a control with a housekeeping mRNA encoding for GAPDH. An immunohistochemical experiment is done to visualize the amounts of Alkaline phosphatase, IGF II, collagen I and fibronectin. Collagen I and fibronectin are both proteins that indicate fibrotic tissue. IGF II is a protein that is involved in the growth of the cell. With this immunohistochemical experiment the elevated amounts of Alkaline phosphatase can be related to higher levels of fibrotic tissue as reflected by collagen I and fibronectin deposition. (6)(7)(8)

Materials and Methods

Alkaline Phosphatase activity assay

Incubation

The 3T3 cells (fibroblasts) were cultivated on 10k, 30k and 100k cells per well. This was done by counting a sample of the incubated cells (x) using a microscope in triplo. X was than multiplied to 10^4, to get the cells/ml suspension. For 24 hours at 37 ºC on a medium consisting of 10% fetal Bovine Serum, pyruvate, and the antibiotics (penicillin and streptomycin). The negative control consisted only out of medium and the positive control being iPLAP (placental alkaline phosphatase) directly pipetted into medium with pNPP.

Stimulation

The 3T3 cells were stimulated with LPS concentrations (100 ng/ml and 300 ng/ml) after the incubation and the activity of the cells was determined after 2 hours and 24 hours.

Alkaline phosphatase activity assay

With each activity assay a standard curve was made with different amounts of pNP and 3T3 medium were mixed up, the table with the exact amounts can be found in *appendix 1*. To get the pNP solution, the pNP was mixed up with 1mM Ammediol buffer. In the experiment the pNPP substrate was 1mM and 50 ul was added to each well containing 80 uL of supernatant of the sample. In the activity assay an 1M ammediol buffer was used together with 0,5 mM MgCl2 to make a reaction buffer. 10 uL of the reaction buffer was added to each well containing a standard curve point. The 96 wells plate was put in the

plate reader at 37 ºC at 405 nm for 30 minutes.
In *appendix 1* a more elaborate description of the assay can be found.

Rupture of cells

The cells were tried to rupture to measure the intracellular matrix using a basic solution to cause a hypertonic environment in the cell. After that the cells were put in -80 ºC multiple times to freeze and melt the water inside the cells. The cells were also sonicated for 20 seconds on 50% amplification in a constant tone and 1 minute in pulse tones at 50% amplification.

Real time PCR with absolute qPCR

Stimulation

The cells were cultivated on a 12 wells plate and were stimulated with LPS (100 ng/ml and 300 ng/ml) for 2 hours and for 24 hours and after that were put in the fridge at -80 ºC.

RNA extraction

For the extraction a Nucleospin (MN) with on-column DNA digestion was used. The 12 wells plate was put on ice from the -80 ºC fridge and a 200 ul homogenization mixture (200 ul of homogenization buffer per well and 20 ul thioglycerol per ml homogenization buffer, so a mixture in 1:50 thioglycerol/homogenization buffer ratio) was put in each well. The cells were scratched of the bottom and so caused a more viscous solution. This viscous solution was transferred into Eppendorf tubes, vortexed and pipetted into the predetermined cartridge slot on the plate.
Special Eppendorf tubes were put in the front slots of the plate with 50 ul of nucleic free water in each tube. The yellow slots of the cartridge were filled with 5 ul of DNases which turns green after the DNases is added. The RNA was extracted into the special Eppendorf tubes in 60 minutes. After that the RNA was stored at -80 ºC.

RNA conversion to cDNA

The RNA samples were put on ice from the -80 ºC fridge and 10 ul of RT mix was added to each of the samples. The conversion of RNA to cDNA was at 20 ºC for 10 min, 42 ºC for 30 min, 99 ºC for 5 min and 20 ºC for 5 min. The cDNA was stored at -20 ºC.

Real time PCR with Absolute QPCR

For the PCR the QuantStudio 7 Flex Real-Time PCR system was used. In *appendix 13* the sequences of the primers used can be found for each protein that is analyzed. Random samples were used to make the standard curve. The forward (20 uL) and reversed primer (20 uL) were mixed to prepare the primer-mix of 10 uM by adding 60 uL of RNase free water to these to primers. The primer-mix were made for GAPDH, AP and TLR4 receptor. The cDNA samples were diluted 10 times. The master mix was prepared per primer using 5,0 uL sybergreen ROX mix, 0,3 uL of the primer-mix and 2,7 ul of H2O per sample. The 384-wells plate was filled with 2 uL for the standard curve points, the samples, and the positive and negative controls in duplo. 8,0 uL of master-mix was then used to fill every used well to a total volume of 10 uL. The PCR was started at 95 ºC for 15 minutes. After that, 35 cycles of 15 seconds at 95 ºC and 1 minutes at 60 ºC.
In *appendix 2* a more elaborate description of the assay can be found.

Immunohistochemical experiment

Preparation of the liver slices

Slices of a healthy and a fibrotic mouse liver were made and dried with a fan for 20 minutes. The slices were treated with acetone for 10 minutes and dried again. The slices were encircled with a dakopen and were rinsed in PBS for 5 minutes three times.

Endogenous peroxidase blocking

The slices were incubated in 0,3% H2O2 in 100% methanol for 20 minutes. Whereafter, the slices were rinsed for 2-3 minutes with water and after that rinsed with PBS for 3 times for 5 minutes.

First antibody incubation

The slices were treated with 50 uL goat antibodies for every tested enzyme and incubated for 60 minutes. After that the slices were rinsed with PBS 3 times for 5 minutes.

Second antibody incubation

The slices were treated with 50 uL rabbit anti-goat antibodies for every tested enzyme and were incubated for 30 minutes. After that the slices were rinsed with PBS 3 times for 5 minutes.

Staining of the slices

The slices were stained with NovaRed kit for a maximum of 10 minutes and after that were washed with H2O.
All the slices were put in a tray to incubate in hematoxylin for 5 minutes and after that were washed with water for 10 minutes and dehydrated with 95% and 100% ethanol for a few seconds.

Staining of slices for Alkaline Phosphatase

A working solution containing Tris-HCl and reagent 1, reagent 2 and reagent 3 (all from the BCIP/NBT kit) in the ratio 9:4:8 were added. A part of the slices was pretreated with acetone and rinsed with Tris-HCl 100 mM. The working solution were added to eats slice (60 uL) and were incubated for a couple minutes in the dark. After the incubation the slices were rinsed with water.
In *appendix 3* a more elaborate description of the assay can be found.

Results

Alkaline Phosphatase activity assay

The alkaline phosphatase activity assay was performed multiple times in different environments. The best number of cells was determined to be 30.000 cells per well. The photos that were made can be found in *appendix 4*. The cells were stimulated with 0, 30, 100 and 300 ng/ml LPS and a positive control with 5 ng/ml TGF-β and a negative control with just the medium of the 3T3 cells. The photos show that not much difference is visible between the stimulated and not stimulated cells. The positive control with TGF-β did result in the formation of a film of cells that did detach from the bottom of the well. To calculate the AP activity of each well a standard curve was made. This standard curve is shown in *figure 2* and it values in *appendix 6*. *Figure 2* shows a R^2 value of 0,9989 and so can be used to calculate the alkaline phosphatase values.

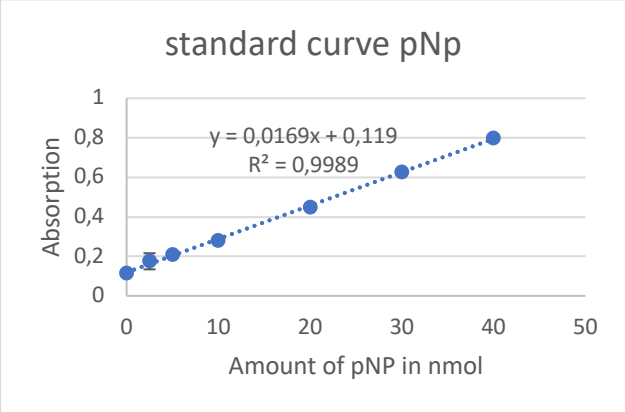

Figure 2: The standard curve of the alkaline phosphatase activity assay using pNP and 3T3 cell medium with on the x-axis the amount of pNP in nmol and on the y-axis the absorption

In *figure 3* the activities of the different cells can be seen on the y-axis of the graph and the concentration of LPS on the x-axis.

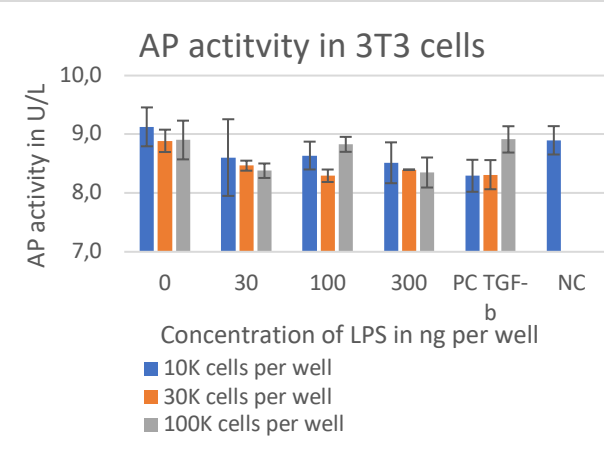

Figure 3: The AP activity in 3T3 cells at different concentrations of LPS on the x-axis and the activity in U/L on the y-axis.

The activities of each of the different numbers of 3T3 cells at their different amounts of LPS used are shown in *appendix 5*. The positive control in the cells is the same or even less than the negative control. However, an iPLAP positive control was also performed in duplo, which gave an activity of 52,27 U/L and so if put in this graph gives a less precise picture of the actual results.

In another experiment the cells the effect of LPS was analyzed after 2 hours and after 24 hours. However, these results were not representable. The photos that were taken during this experiment are shown in *appendix 7.* These photos show that the amount of LPS has very little effect on the morphology of the cells. The cells seem to be denser in the 24 hours of LPS stimulation compared to the 2 hours stimulation.

Real time PCR with absolute qPCR

A qPCR was done on three different enzymes namely, the housekeeping gene GAPDH to test if the samples give an expected result and the enzyme presence of Alkaline Phosphatase and the TLR4 receptor. The standard curve was made of each enzyme. The efficiency was determined for each of these standard curves to determine the how representable the information is achieved from the standard curve. Points can be determined as insignificant when the melting plot is does not show the expected curve. Furthermore, the amplification curve was made as well for each sample.

GAPDH

The GAPDH standard curve is shown in *figure 4* and has a slope of -5,1418. The efficiency of the standard curve was calculated at 94,5% (Ef= -1+10^(-1/slope)).

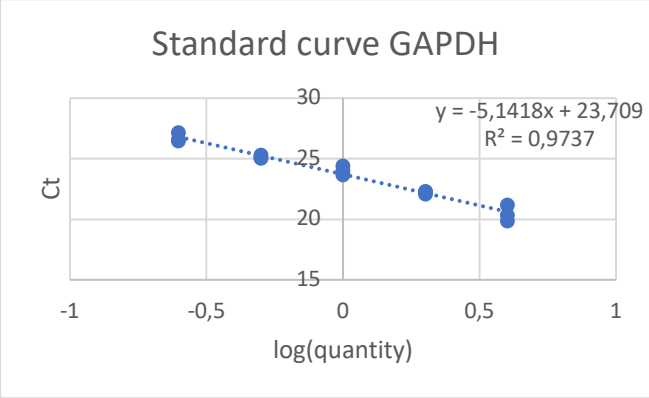

Figure 4: Standard curve of the qPCR of GAPDH with on the x-axis the log(quantity) and on the y-axis the Ct value.

The amplification curve and the melting curve plot of the standard curve and the samples of GAPDH are shown in *appendix 8*. The melting curve plot show that the data obtained from the GAPDH are suitable to use in the standard curve. In *appendix 9* the quantity of the samples is calculated.

Alkaline Phosphatase and TLR4 receptor

The standard curves of the qPCR of both alkaline phosphatase and the TLR4 receptor are shown in *figure 5.* The red dots indicate the points of AP, and the green dots represent the points of the TLR4 receptor.

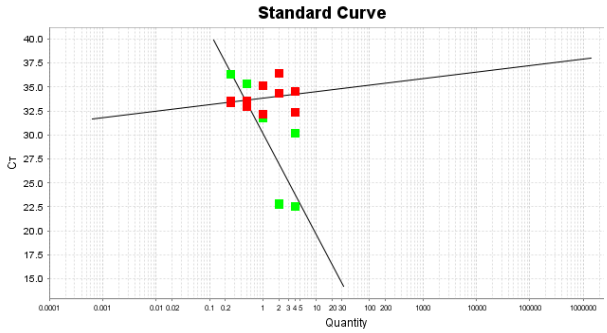

Figure 5: The standard curves of the qPCR of AP (red) and TLR4 receptor (green). The standard curve with the positive slope is that of AP, the other strongly negative one is that of TLR4 receptor. On the x-axis the quantity is shown on a logarithmic scale and the y-axis shows the Ct value.

The slope of the AP standard curve is 0,681 and the slope of the TLR4 receptor standard curve is -10,525. This gives an efficiency of -96,6% for AP and 24,5% for TLR4 receptor. This is not sufficient to support any statements about the amounts of mRNA found in the cells that encode for AP or TLR4 receptor. The amplification curves and the melting curves accompany this outcome since these are not consistent as well. Both the amplification curves and the melting curves can be found in *appendix 10 and 11.*

Immunohistochemical experiment

After the staining as described in the materials and methods of this experiment the liver cells were put under a microscope and pictures were taken. These pictures are shown in *appendix 12.* The antibody staining is encircled with a green circles, the blue circles indicate the central veins and the red circles indicate the portal veins. The pictures of the healthy liver showed only the purple stain that is caused by hematoxylin. The diseased liver slices all showed red (NovaRed) stains through the slices. The slices that are used for alkaline phosphatase staining all show a red colour in the diseased liver cells and show similar staining as that of IGF-II and Collagen I. The diseased slices that are stained on AP show typical fibrotic tissue compared to the healthy livers.

Discussion

In current studies the elevated amounts of LPS in blood and organs often are the cause of serious diseases like sepsis or chronic pulmonary or gastrointestinal infections. (9) LPS attaches to the TLR4 receptor and triggers a cascade of reactions. These inflammatory responses cause the stellate cells in the liver to differentiate and start producing extracellular matrix. (5) The TLR4 receptor can be inhibited and so the cascade can be prevented. This is done with Ibudilast, a TLR4 receptor antagonist. (10) Alkaline phosphatase is known to inactivate LPS and therefore could be an alternative solution to the problem. Therefore, in this experiment the aim was to study if different levels induced higher levels of Alkaline Phosphatase and if this has any effect on fibrosis of the tissue. With an alkaline phosphatase activity assay the amount of pNPP transformed into pNP was measured in U/L. AP is known to convert pNPP into pNP. The results, shown in *appendix 5,* show that the negative control of the experiment, consisting of the medium of the 3T3 cells, produces a significant signal of 8,89 U/L. The different levels of LPS used to stimulate the 3T3 cells in producing Alkaline Phosphatase did not have any effect on the activity. This means that there was no alkaline phosphatase present to convert pNPP into pNP or the alkaline phosphatase was not able to convert the substrate. The positive control done with iPLAP pipetted directly on the substrate does show a high response of 52,27 U/L and so the experiment is valid. In another experiment that was carried out the cells were tried to be ruptured to see if AP levels were rising intracellularly. However, the cells were very hard to rupture and so the intracellular AP was difficult to reach. The photos of the cells after 2 hours and 24 hours of stimulation with LPS, shown in *appendix 7,* show that the cells still are dividing, but the 24 hours

do show more granulocytes than the 2 hours photos. Therefore, can be stated that the LPS is inducing an inflammatory response in the cells.

To support this statement a qPCR was done to look at mRNA levels to the proteins that cause this inflammatory response. The qPCR was done of the GAPDH mRNA which is a housekeeping protein and should be present in similar amounts in every cell regardless of the amounts of LPS used to stimulate the cells. The mRNA of AP and TLR4 receptor were used to perform another qPCR to determine the elevated levels these mRNAs after the stimulation with LPS and so the start of the inflammatory response.

The GAPDH qPCR standard curve gave an efficiency of 94,5% and a R^2 value of 0,974. The GAPDH qPCR results, shown in *appendix 8 and 9.* It shows results at 2 hours of stimulation all around the quantity of 1 regardless of the amount of LPS to stimulate. The standard deviations all are very high and so the reproducibility of this experiment is questionable. The 24 hours quantities show some quantities around 1, but also a very low quantity at 300 ng/ml of LPS. The positive control is much higher than that of the 2 hours of stimulation and therefore cannot be valid. When looking at the amplification curve and the melting plot of the GAPDH qPCR, most of the samples seems to give valid results and only a few results need to be excluded. The AP and TLR4 receptor qPCRs are however even less valid. The standard curves efficiencies were respectively -96,6% and 24,5%. This means that the standard curves cannot be used to determine the quantities of mRNA present in the samples. When looking at the melting plot and the amplification curve of AP, *appendix 10,* and of TLR4 receptor, *appendix 11,* there can be stated that the experiment did fail and had many contaminations in it. All the qPCR results were produced out of the same mRNA samples that were created when the mRNA was extracted and converted. Contaminations or interference with RNases could have played an important role in the results that came from these experiments.

An immunohistochemical staining experiment was done to determine the presence of elevated levels of alkaline phosphatase in a fibrotic mouse liver compared to a healthy mouse liver. The proteins IGF-II, collagen I and fibronectin are also involved in the fibrosis in a liver, collagen I and fibronectin both with the formation of extracellular matrix and IGF-II induces the formation of myofibroblasts and so indirectly the formation of extracellular matrix. (6)(7)(8) The results showed in *appendix 12* show that the negative control with only PBS shows that there is no staining of the NovaRed, the purple colour is caused by the hematoxylin. When looking at collagen I the fibrotic liver shows red staining close to the cell wall and follow the path from the vena porta to the central vein through the hepatocytes. The results of the IGF-II stained cells show much less staining than that of collagen I. However, it still shows staining around the cell walls and accompanies the typical round shaped tissue that is formed when a liver is fibrotic. The results of fibronectin-stained cells show a very strong response, but the healthy cells do to. A too high concentration was used in this experiment, where the antibodies were diluted 1:500 instead of 1:1000. This resulted in a bit of overstaining, but the results however show that the fibrotic liver slices have more staining. The Alkaline Phosphatase staining shows a very clear staining of the fibrotic liver slice compared to the healthy liver. Like the previous experiments on collagen I and fibronectin the staining is most abundant in the borders of the tissue that is close to the passage that connects the vena porta and the central vein. From these

results there can be stated that the Alkaline phosphatase is present in higher levels in fibrotic liver compared to the healthy liver and shows that it is present in similar areas as the proteins collagen I and fibronectin. This indicates that alkaline phosphatase is closely related to the fibrosis of a liver.

Conclusion

All in all, the alkaline phosphatase activity assay, qPCR and immunohistochemical experiment did not give the expected results. The activity assay showed no increasing amounts of activity when an increasing amount of LPS was added to the cells. In the experiment the attempt to rupture the cells failed multiple times and so the intracellular alkaline phosphatase activity could not be measured. The results of the qPCR of GAPDH did show promising results when looking at the standard curve points but fewer clear assumptions were made from the quantities of the samples. The qPCRs of the RNA that encodes for the protein alkaline phosphatase and the TLR4 receptor showed standard curves with low efficiency and melting plots and amplification curves which were not representable to draw conclusions from. The immunohistochemical assay did show clear results that alkaline phosphatase is involved in fibrotic liver tissue. The proteins collagen I and fibronectin both showed similar results and IGF-II showed some increase as well but suffered a bit from overstaining.

In future studies the cells could be tried to rupture with a longer sonication or with a more basic solution for a longer period to determine any intracellular AP. Moreover, in normal livers the process of fibrosis takes years to develop. The stimulation could be done on 3T3 cells could be tested after a week instead of just 2 or 24 hours. (12) The qPCR of AP and TLR4 receptor did fail and therefore should be repeated with new RNA extracted samples, to ensure that RNases did have no effect on the samples. The RNA sequence coded for LPS binding protein could also be analyzed in qPCR for a more detailed analysis. ELISA studies could be done as well to get a more quantitative analysis of the immunohistochemical experiment. ELISA uses labelled antibodies that bind to the specific protein with a colour change in the wells when the protein is bound to the labelled antibodies an amount of this protein can be calculated. Another technique that could be used in future studies is FACS. In this case the cells that express alkaline phosphatase can be sorted so that further studies on those cells can be done. For example, the increase in levels of other pro-inflammatory cytokines. (13)(14)

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Appendices

Appendix 1: Alkaline phosphatase activity assay

Incubation

The 3T3 cells were extracted from a vessel where the cells were grown in using trypsin The cells were cultivated in 10.000 cells per well, 30.000 cells per well and 100.000 cells per well to determine the optimal number of cells per well. The cells were then put in the incubator for at 37 ºC for 2 hours and 24 hours. The negative control consisted only out of medium of the 3T3 cells and the positive control being iPLAP (placental alkaline phosphatase) directly pipetted into medium with pNPP.

Stimulation

The 3T3 cells were stimulated with LPS concentrations (100 ng/ml and 300 ng/ml) after the incubation and where the activity of the cells were determined after 2 hours and 24 hours.

Activity assay

With each activity assay a standard curve was made with different amounts of pNP and 3T3 medium were mixed up, the table with the exact amounts can be found in *appendix 1*. To get the pNP solution, the pNP was mixed up with 1mM ammediol buffer. In the experiment the pNPP substrate was 1mM and 50 ul was added to each well containing 80 uL of supernatant of the sample. In the activity assay an 1M ammediol buffer was used together with 0,5 mM MgCl2 to make a reaction buffer. 10 uL of the reaction buffer was added to each well containing a standard curve point. The 96 wells plate was put in the plate reader at 37 ºC at 405 nm for 30 minutes.

Table 1: The amounts to pipet to get the standard curve in triplo.

Standard (nmol)	**1 mM pNP (uL)**	**Medium (uL)**	**Vt (uL)**
40	120	780	900
30	90	810	900
20	60	840	900
10	30	870	900
5	15	885	900
2,5	7,5	892,5	900
0	0	900	900

Appendix 2: Real time PCR with absolute qPCR

Stimulation

The cells were cultivated on a 12 wells plate and were stimulated with LPS (100 ng/ml and 300 ng/ml) for 2 hours and for 24 hours and after that were put in the fridge at -80 ºC.

RNA extraction

- Get the cells out of the -80 degrees fridge and put them in an iced container.
- Put gloves on and try to touch as little as possible since RNases is present on your body which can interfere with the results.
- Make the homogenization buffer where your cells are later pipetted in. 200 ul of homogenization buffer per sample.
- To complete the homogenization buffer, add 20 ul of thioglycerol (do this very slowly since this is a highly viscous fluid which is difficult to pipette) per ml homogenization buffer to the homogenization buffer.
- Prepare 1 normal Eppendorf tube and 1 special one for in the plate for each sample.

- Fill the plate with cartridges
- Carefully put 200 ul of the homogenization mixture into each sample well. And scratch the bottom with the tip of the pipette to loosen the broken cells and get them in the mixture. After some scratching you should see a more viscous slimy fluid than before which means that the cell mixture is in now mixed with the fluid.
- Suck every bit of cell homogenization buffer mixture out of the well into its normal Eppendorf tube and vortex for 15 seconds.
- After the vortex pipette it into the top slot of the cartridge.
- Do this for every sample.
- Fill the special tubes with 50 ul of nucleic free water on the bottom of the tube.
- Fill the yellow slots of the cartridge with 5 ul of DNases, which should turn green when DNase is added.
- Put the plate in the reader and run the test.
- Store the RNA at -80 ºC.

RNA conversion to cDNA

The RNA samples were put on ice from the -80 ºC fridge and 10 ul of RT mix was added to each of the samples. The conversion of RNA to cDNA was at 20 ºC for 10 min, 42 ºC for 30 min, 99 ºC for 5 min and 20 ºC for 5 min.

Precaution:
Tubes, tips, and water must be RNase free.
Use gloves to prevent RNase going into the solution.

RT mix per sample:
See *table 2* on how to make RT mix per sample.

Table 2: The substances needed for the RT mix per sample.

RT buffer	2,0 uL
dNTP (=A, G, C, T) mix (25 mM)	0,1 uL
RNasin	0,25 uL (=10 units)
Rev Transcriptase	0,5 uL (=100 units)
Random hexamers	0,5 uL (=0,5 ug)
RNA	0,5 ug (preferably in 5 uL)
H2O	1,65 uL (to get the total of 10uL)
Total volume	10 uL

Converting RNA to cDNA:
10 min 20 ºC
30 min 42 ºC
5 min 99 ºC
5 min 20 ºC

Real time PCR with Absolute QPCR

1. The random samples of the cDNA for the standard curve need to be prepared according to *table 3.*

Table 3: Standard curve points of the cDNA samples mixed.

Standard curve concentrations	cDNA mix of all samples	RNase free water
4	120 uL (12*10 uL)	180 uL
2	120 uL of **4**	120 uL
1	120 uL of **2**	120 uL
0,5	120 uL of **1**	120 uL
0,25	120 uL of **0,5**	120 uL
NC	-	120 uL

2. Prepare your primer-mix:
   Prepare the primer stocks each to a final concentration 50 uM. With these get the primer-mix of 10 uM by adding: 20 uL of forward primer + 20 uL of reversed primer + 60 uL of RNase free water.
3. Dilution of the cDNA samples: 10 uL of cDNA + 90 uL of H2O
4. Prepare the PCR master mix per primer:
   Sybergreen ROX mix 5,0 uL
   Primer-mix (10 uL) 0,3 uL
   H2O 2,7 uL
   Total volume 8,0 uL
5. Pipet the following amounts on the 384 wells plate.
   a. Pipet 2,0 uL of standards into the plate.
   b. Pipet 2,0 uL of NC and PC in duplo per primer.
   c. Pipet 2,0 uL of diluted cDNA samples in the plate.
   d. Add 8,0 uL of PCR master mix per well.
6. Seal of the 384 wells plate
7. Run the real time PCR:

Stage 1: 15 minutes at 95 ºC.
Stage 2: 15 seconds at 95 ºC.
1 minutes at 60 ºC.
Dissociation protocol for melting curve at 60 ºC needs to be turned on.

Appendix 3: Immunohistochemical experiment

Immunohistochemical staining with NovaRed

- Dry sections for 20 minutes with a fan
- Fixation with acetone for 10 minutes (max. 15 minutes)
- Dry the sections on the air
- Draw circle around the sections with a dakopen. (Let the circle dry)
- Rinse in PBS for 5 minutes (3 times with new PBS)

Endogenous peroxidase blocking:

- Incubate in 0,3% H_2O_2 in 100% methanol (0,6 ml H_2O_2 in 60 ml MeOH) for 20 minutes
- Rinse with water for 2-3 minutes.
- Rinse in PBS for 5 minutes (3 times with new PBS)

First antibody treatment (goat antibody):

- 50 uL of the antibody per slice for 60 minutes
- Rinse in PBS for 5 minutes (3 times with new PBS)

Second antibody treatment (rabbit anti-goat antibody):

- 50 uL of the antibody per slice for 30 minutes
- Rinse in PBS for 5 minutes (3 times with new PBS)

- Staining with NovaRed kit for max 10 minutes
- Wash the slice with water

- Incubate for 5 minutes in hematoxylin
- Rinse in water for 10 minutes.
- Dehydrate in 95% ethanol and after that in 100% ethanol for a few seconds
- Dry the slices and cover them using a cover glass

BCIP/NBT alkaline substrate kit IV

Working solution preparation:

1. Prepare 100mM Tris-HCl pH 9,5 (approximately 100 ml, most of it used for washing the slice)
2. Use 60 uL per slice of Tris-HCl
3. Add 18 uL reagent 1 per ml buffer and vortex
4. Add 8 uL reagent 2 per ml buffer and vortex
5. Add 16 uL reagent 3 per ml buffer and vortex

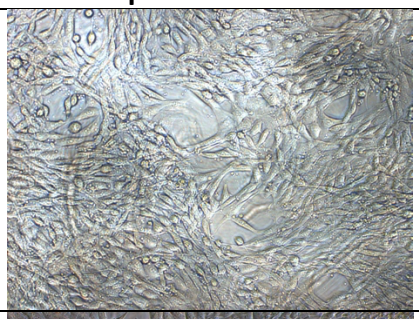
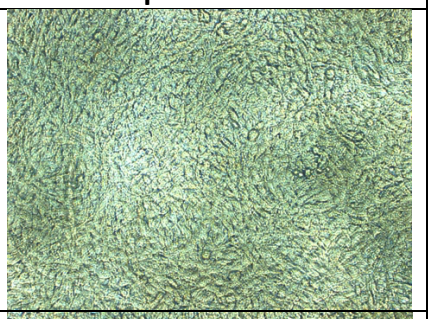
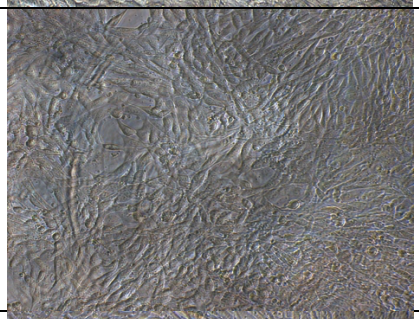
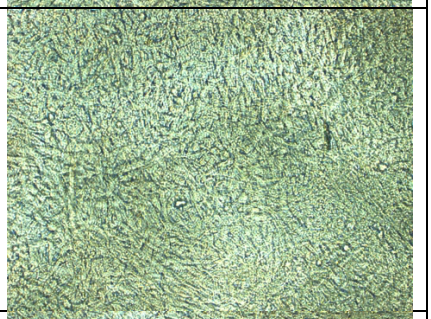
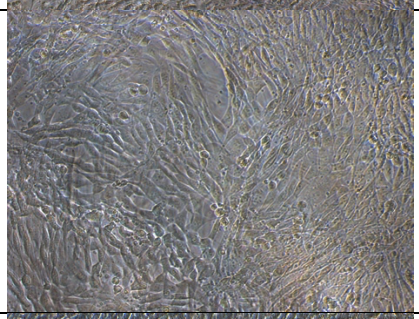
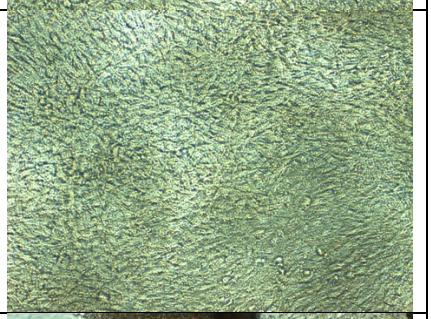
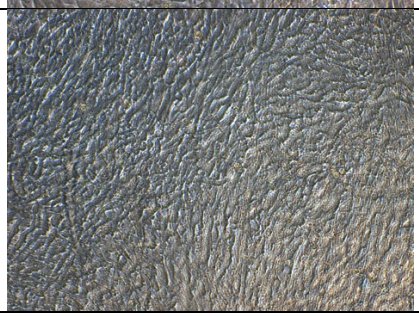
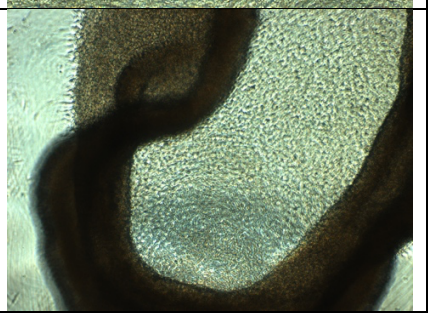
Incubation:

1. Pipet 60 uL of working solution and incubate for several minutes in dark
2. Wash section in 100 mM Tris-HCl pH 9,5 for 5 minutes
3. Rinse with tap water
4. Dry slices and cover them using a cover glass.

Appendix 4: Photos of the cells after stimulation with LPS

These pictures represent the cells after stimulation with 100 ng/ml LPS, 300 ng/ml and 5 ng/ml TGF-β. The 100.000 cells per well are much more populated and at the positive control with TGF-β the cells even formed a film and detached from the bottom of the well.

Table 4: Pictures made with a microscope of the different density and amounts of LPS added and with the positive control with TGF-β.

	30k cells per well	**100k cells per well**
3T3 0 ng/ml LPS		
3T3 100 ng/ml LPS		
3T3 300 ng/ml LPS		
3T3 5 ng/ml TGF-β		

Appendix 5: The Alkaline phosphatase activity for the different number of cells per well stimulated with different amounts of LPS.

Table 5: The AP activity for the different LPS concentrations with a positive control in the 10.000 cells per well.

	0 ng/ml LPS	30 ng/ml LPS	100 ng/ml LPS	300 ng/ml LPS	5 ng/ml TGF-β
average mili units slope	12,00	11,67	11,72	11,55	11,26
nmol pnp (mili units)	707,8	688,3	691,1	681,2	663,5
units	0,7078	0,6883	0,6911	0,6812	0,6635
µmol pnp (µmol/min so U)	0,00071	0,00069	0,00069	0,00068	0,00066
U/L	8,847	8,604	8,638	8,515	8,294
Standard deviation in U/L	0,331	0,653	0,237	0,348	0,272

Table 6: The AP activity for the different LPS concentrations with a positive control in the 30.000 cells per well.

	0 ng/ml LPS	30 ng/ml LPS	100 ng/ml LPS	300 ng/ml LPS	5 ng/ml TGF-β
average mili units slope	12,06	11,49	11,26	11,40	11,28
nmol pnp (mili units)	711,1	677,3	663,6	671,8	665,0
units	0,7111	0,6773	0,6636	0,6718	0,6650
µmol pnp (µmol/min so U)	0,00071	0,00068	0,00066	0,00067	0,00067
U/L	8,889	8,466	8,295	8,398	8,313
Standard deviation in U/L	0,190	0,085	0,106	-0,006	0,248

Table 7: The AP activity for the different LPS concentrations with a positive control in the 100.000 cells per well.

	0 ng/ml LPS	30 ng/ml LPS	100 ng/ml LPS	300 ng/ml LPS	5 ng/ml TGF-β
average mili units slope	12,08	11,37	11,98	11,33	12,09
nmol pnp (mili units)	712,2	670,4	706,3	668,0	713,1
units	0,7122	0,6704	0,7063	0,6680	0,7131
µmol pnp (µmol/min so U)	0,00071	0,00067	0,00071	0,00067	0,00071
U/L	8,903	8,380	8,829	8,350	8,913
Standard deviation in U/L	0,330	0,123	0,127	0,256	0,224

Table 8:The AP activity for the negative control and the positive control with iPLAP

	NC	PC (iPLAP)
average mili units slope	12,07	70,35
nmol pnp (mili units)	711,8	4181,6
units	0,7118	4,1816
µmol pnp (µmol/min so U)	0,00071	0,00418
U/L	8,898	52,270
Standard deviation in U/L	0,241	1,023

Appendix 6: Standard curve of pNP with 3T3 medium.

The results of the standard curve of pNP with the 3T3 medium can be seen in *table 8*.

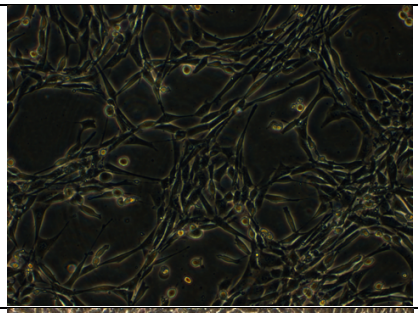
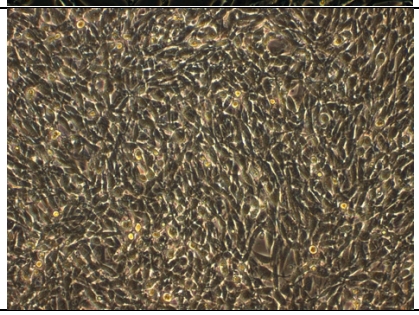
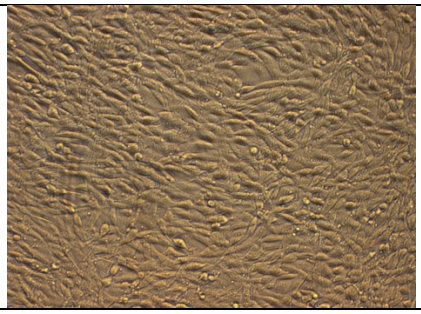
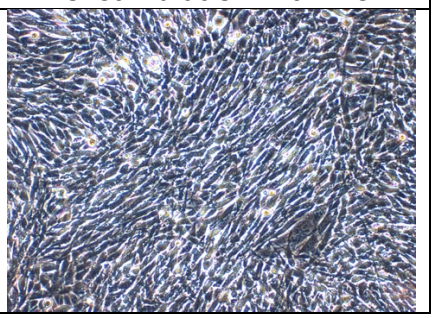
Table 9: Results of the standard curve of pNP with the 3T3 medium.

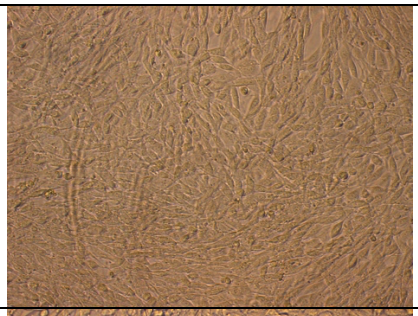
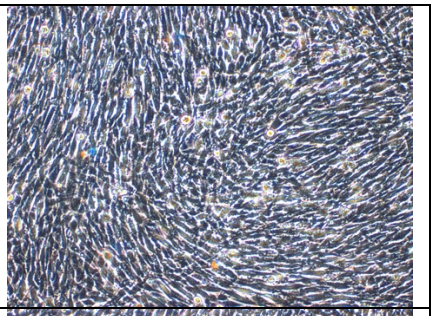
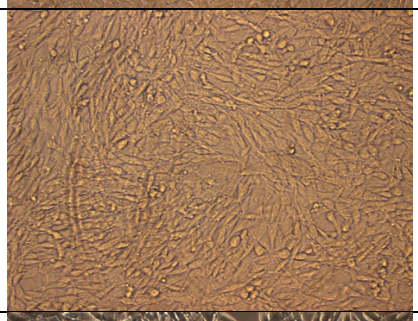
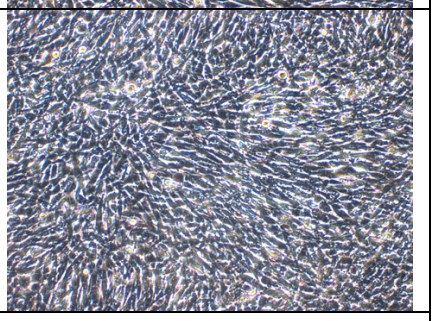
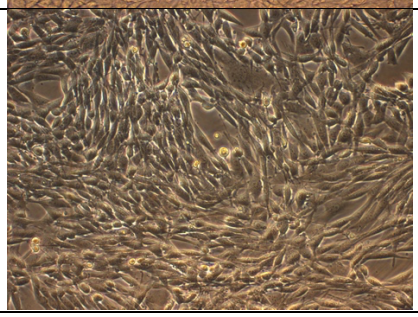
1st absorption	2nd absorption	Average	Nmol pNP	Standard deviation
0,099	0,104	0,10	0	0,0035
0,149	0,151	0,15	2,5	0,0014
0,219	0,22	0,22	5	0,0007
0,273	0,288	0,28	10	0,0106
0,442	0,434	0,44	20	0,0057
0,623	0,621	0,62	30	0,0014
0,783	0,78	0,78	40	0,0021

Appendix 7: Photos of the second experiment with the LPS stimulation after 2h and after 24h.

These pictures represent the cells after stimulation with 100 ng/ml LPS, 300 ng/ml and 5 ng/ml TGF-β at 2 hours after stimulation and 24 hours after stimulation. 40.000 cells per well were used.

Table 10: Photos made with a microscope before and after the incubation and after 2 hours and 24 hours of stimulation with LPS and the positive control with TGF-β.

Before incubation		
After incubation (24h)		
	2h of stimulation with LPS	**24h of stimulation with LPS**
3T3 0 ng/ml LPS		

3T3 100 ng/ml LPS		
3T3 300 ng/ml LPS		
3T3 5 ng/ml TGF-β		**No photo taken.**

Appendix 8: melting plot and amplification curve of GAPDH

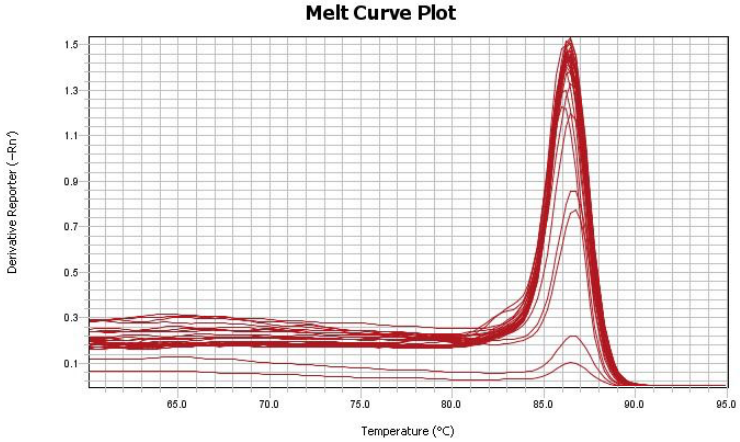


Figure 6: The Melt curve plot for each sample and standard curve point with on the x-axis the temperature in °C and on the x-axis the derivative reporter in (-Rn)

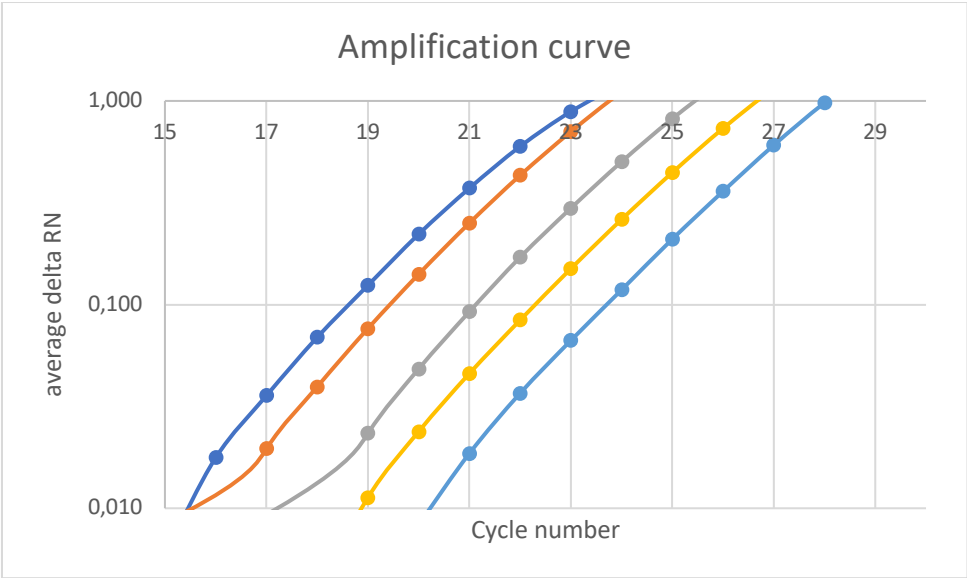


Figure 7: The amplification curve of the standard curve points of GAPDH.

Appendix 9: Calculated quantities of GAPDH in the samples.

Table 11: The calculated quantities of GAPDH samples with the different amounts of LPS and a positive control with TGF-β after 2 hours of stimulation.

Samples at 2 hours	**Quantity**	**Standard deviation**
0 ng/ml LPS	0,810	0,556
100 ng/ml LPS	0,954	1,348
300 ng/ml LPS	1,521	0,518
5 ng/ml TGF-β	0,587	0,824

Table 12: The calculated quantities of GAPDH samples with the different amounts of LPS and a positive control with TGF-β after 24 hours of stimulation. The 300 ng/ml LPS stimulation had only one value and so the standard deviation cannot be calculated.

Samples at 24 hours	**Quantity**	**Standard deviation**
0 ng/ml LPS	0,643	0,906
100 ng/ml LPS	2,058	0,401
300 ng/ml LPS	0,013	-
5 ng/ml TGF-β	144,586	200,752

Appendix 10: The amplification curve and the melting curve for both the standard curve points and the samples of the AP qPCR

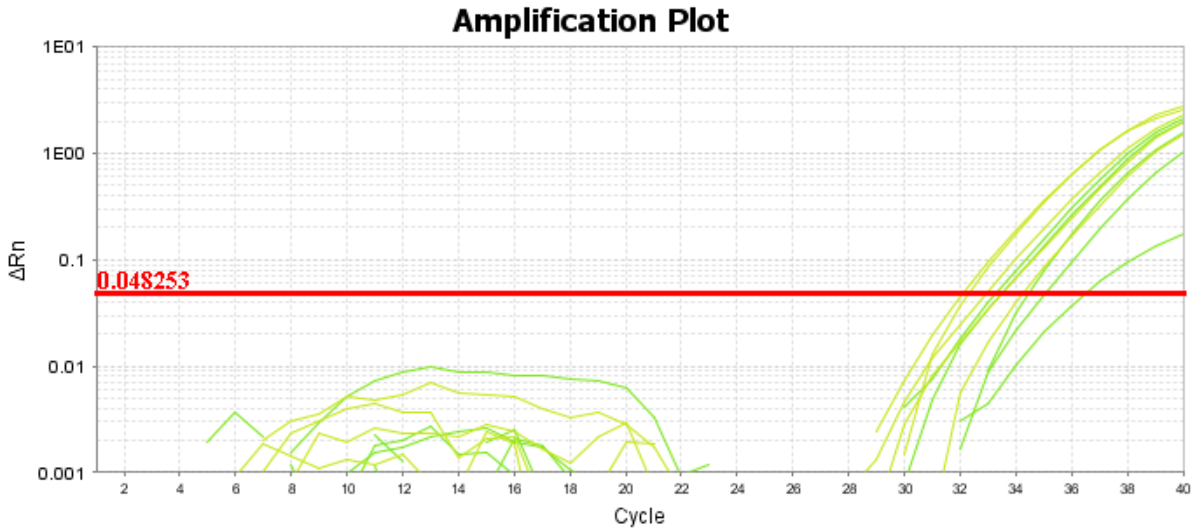


Figure 8: The Amplification plot of the standard curve points of the AP qPCR with on the x-axis the number of cycles and on the y-axis the ΔRn.

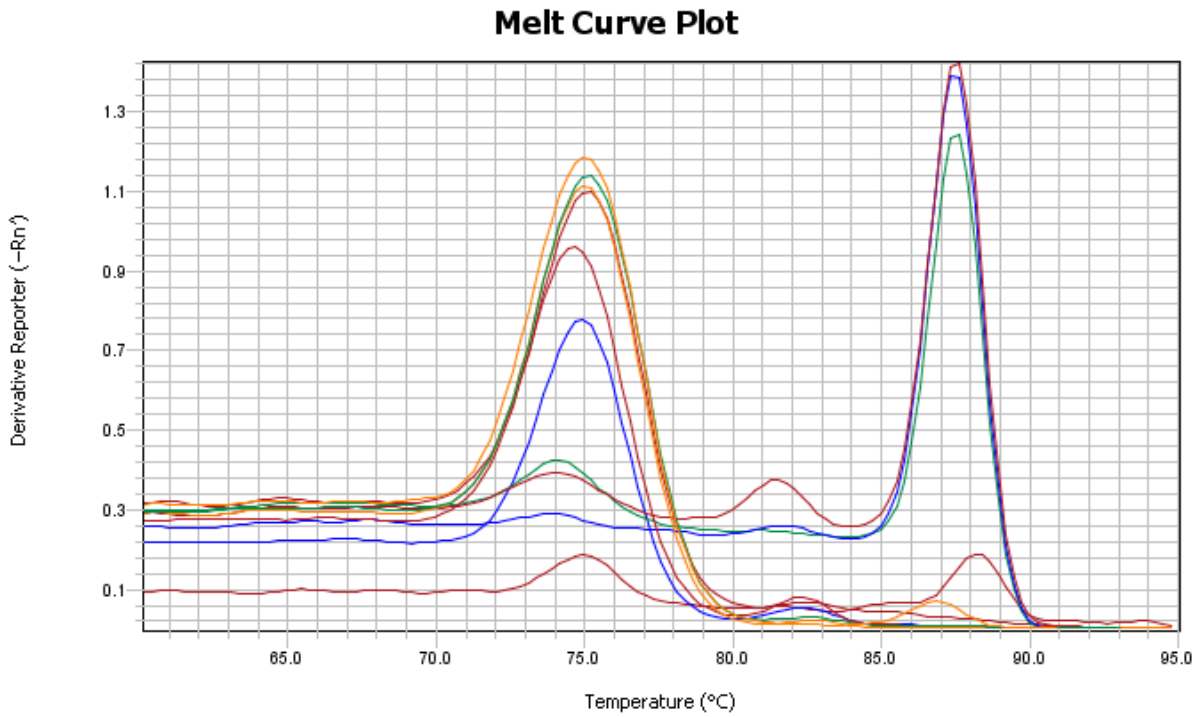


Figure 9: The melt curve plot of the standard curve points of the AP qPCR with on the x-axis the temperature in °C and on the y-axis the derivative reporter in -Rn.

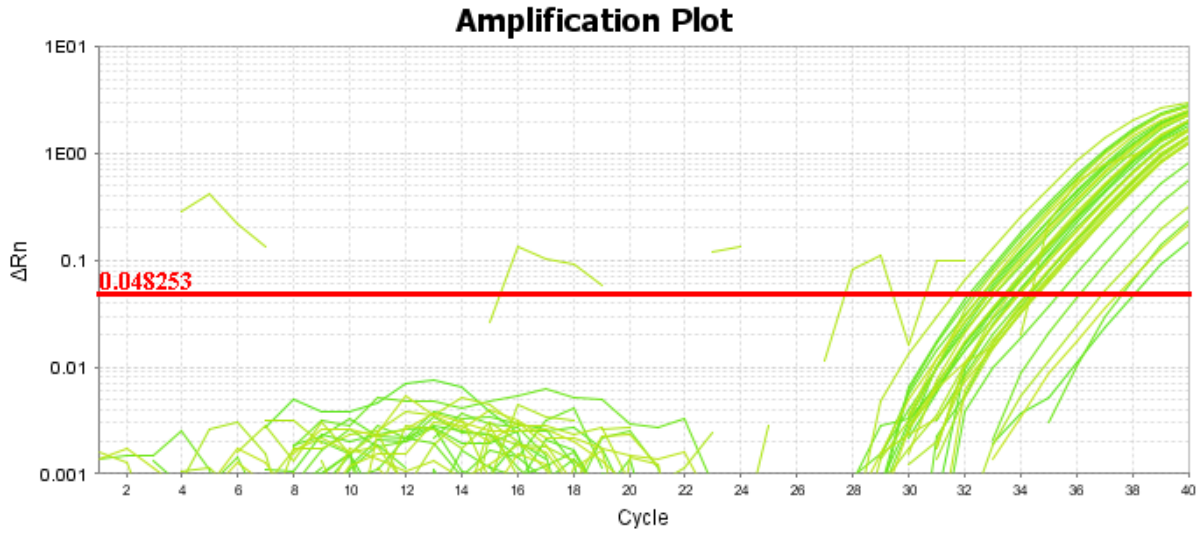


Figure 10: The Amplification plot of the samples of the AP qPCR with on the x-axis the number of cycles and on the y-axis the ΔRn.

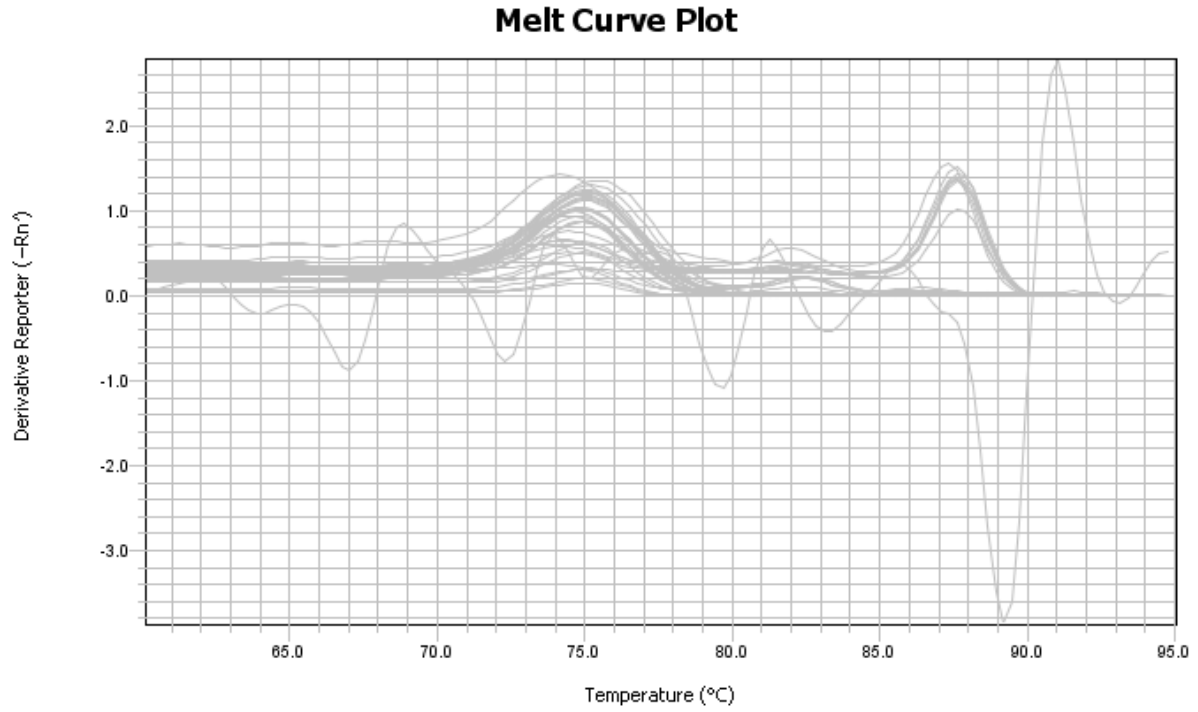


Figure 11: The melt curve plot of the samples of the AP qPCR with on the x-axis the temperature in °C and on the y-axis the derivative reporter in -Rn.

Appendix 11: The amplification curve and the melting curve for both the standard curve points and the samples of the TLR4 receptor qPCR

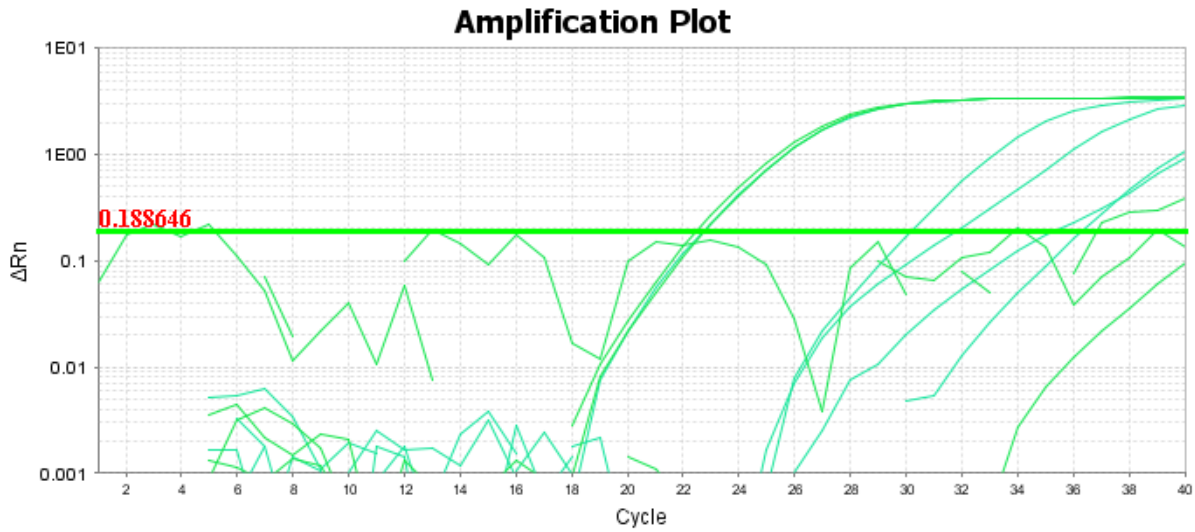


Figure 12: The Amplification plot of the standard curve points of the TLR4 receptor qPCR with on the x-axis the number of cycles and on the y-axis the ΔRn.

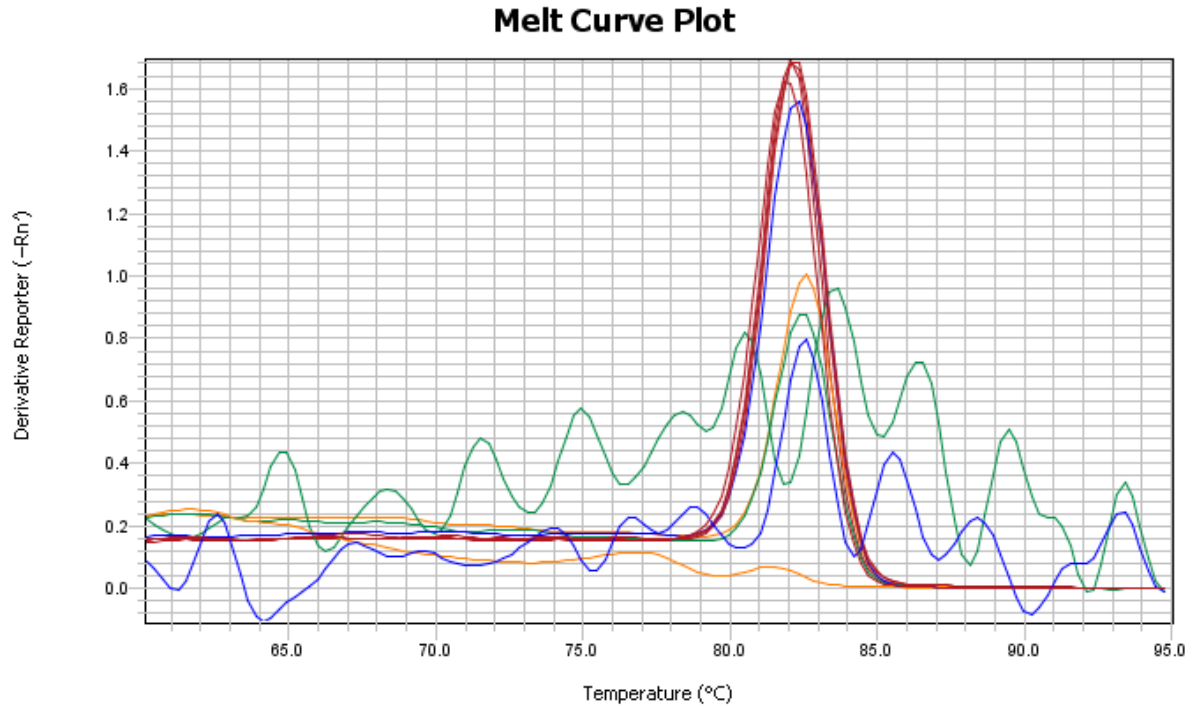


Figure 13: The melt curve plot of the standard curve points of the TLR4 receptor qPCR with on the x-axis the temperature in °C and on the y-axis the derivative reporter in -Rn.

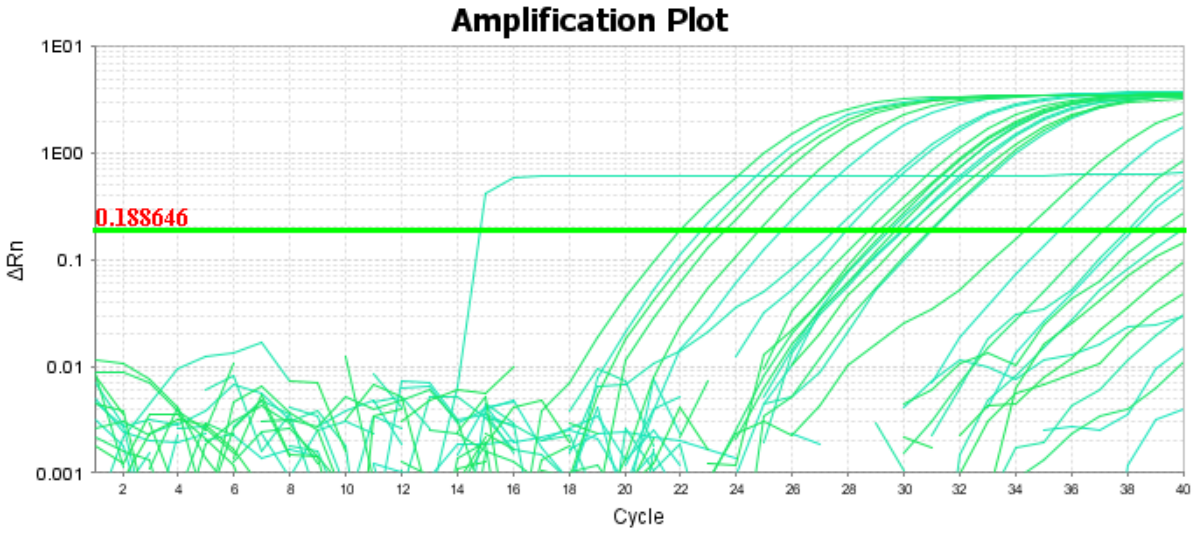


Figure 14: The Amplification plot of the samples of the TLR4 receptor qPCR with on the x-axis the number of cycles and on the y-axis the ΔRn.

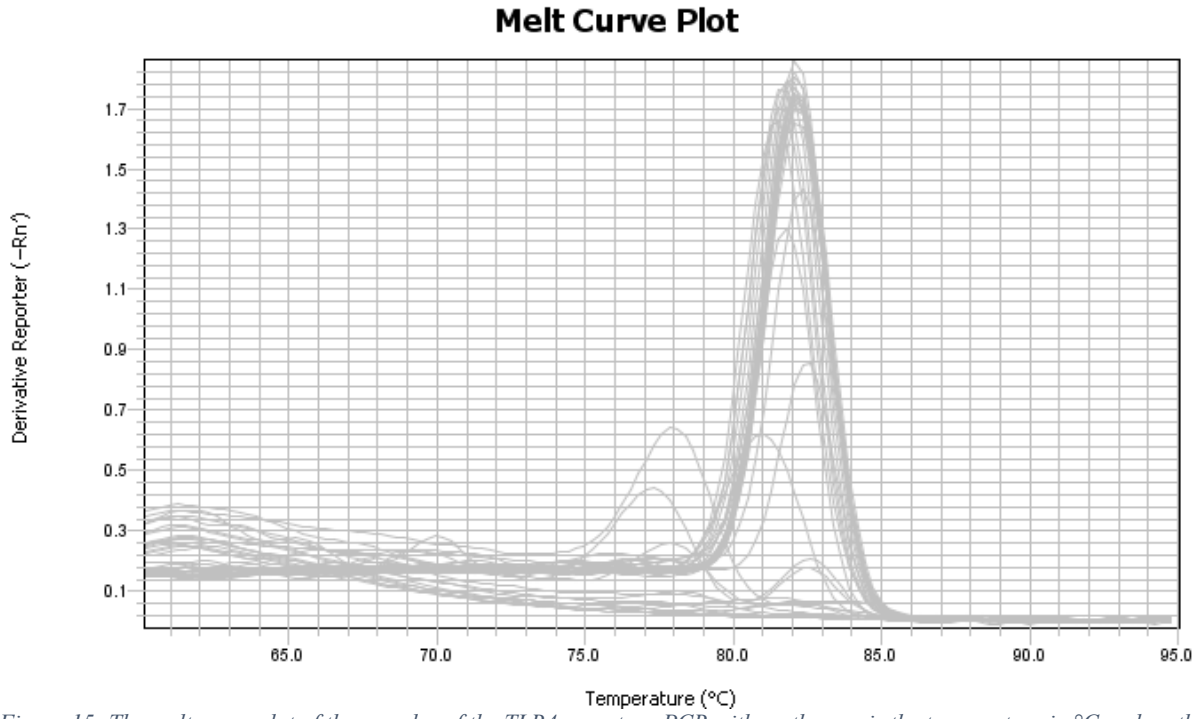


Figure 15: The melt curve plot of the samples of the TLR4 receptor qPCR with on the x-axis the temperature in °C and on the y-axis the derivative reporter in -Rn.

Appendix 12: Pictures of the stained liver slices for the immunohistochemical experiment.

Table 13: photos made with a microscope of the immunohistochemical experiment of cells tested with an antibody on IGF-II, Fibronectin, and collagen I and a negative control with PBS of healthy and fibrotic liver slices. The red circles represent the portal vein area, the blue circles the central vein area and the green circles the stained area.

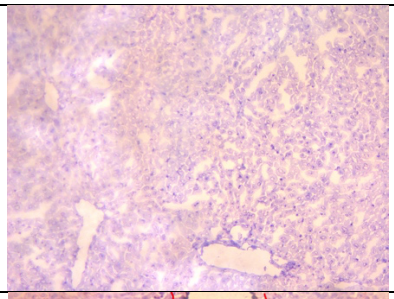
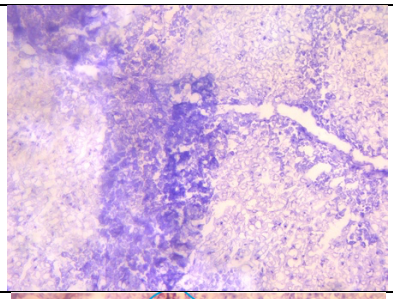
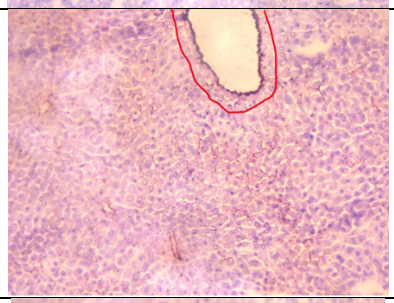
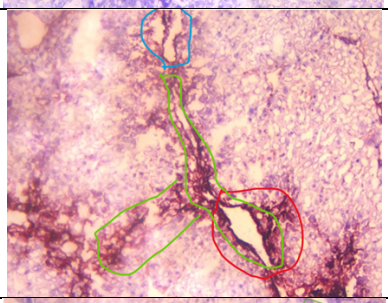
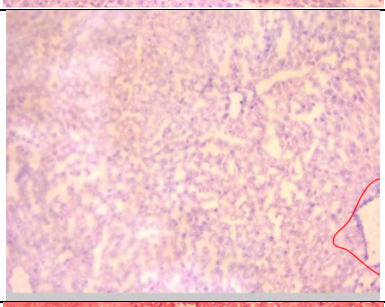
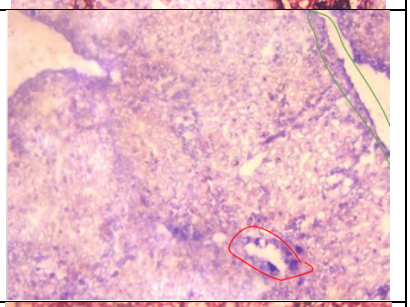
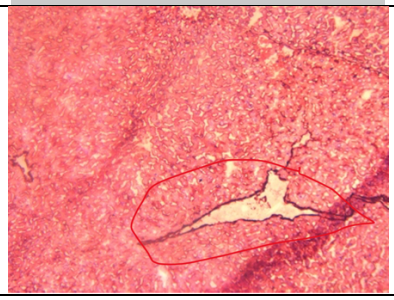
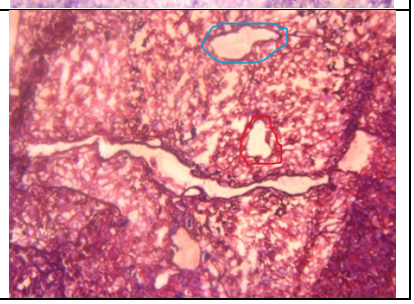
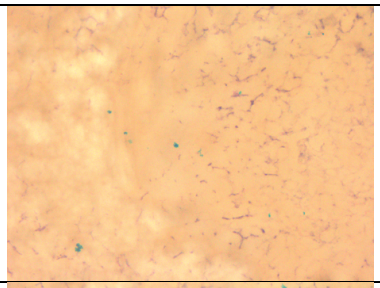
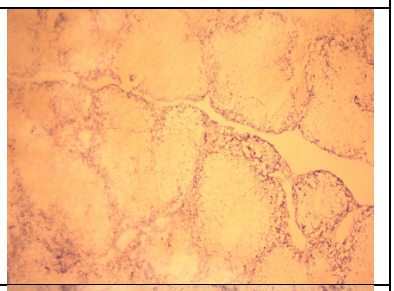
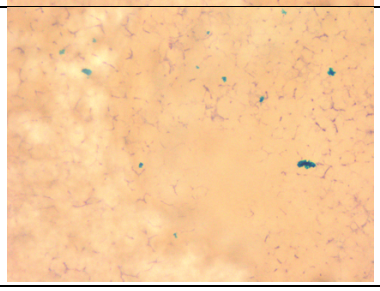
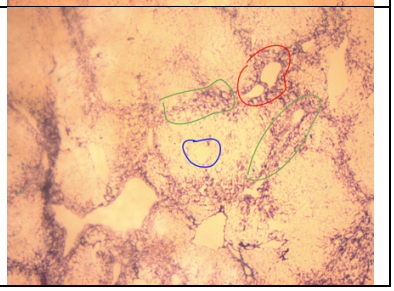
	Healthy	diseased
PBS		
Collagen I		
IGF-II		
Fibronectin		

Table 14: Photos made with a microscope of the immunohistochemical experiment with an antibody for alkaline phosphatase on healthy and diseased cells (fibrotic).

	Healthy	**Diseased**
Alkaline Phosphatase (PBS)		
Alkaline Phosphatase (Acetone)		

Appendix 13: The nucleotide sequence of the primers of the analyzed proteins.

Table 15: Forward and reversed primer nucleotide sequences used for the qPCR.

Primer sequences	**Forward**	**Reversed**
GAPDH	5'-ACAGTCCATGCCATCACTGC	5'-GATCCACGACGGACACATTG
AP	5'-TAACACCAACGCTCAGGTCC	5'-GTAGTCACAATGCCCACGGA
TLR4 receptor	5'-GAGTGCCCCGCTTTCACCTCT	5'-GCCAGAGCGGCTGCTCAGAAA