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VALIDATION OF ANALYTICAL METHODS
FOR THE DETECTION OF CYTOKINES IN
THE EARLY STAGES OF HEART FAILURE

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List of abbreviations

ANP: Atrial Natriuretic Peptide
AU: Absorbance Units
BNP: Brain Natriuretic Peptide
CNP: C-type Natriuretic Peptide
ELISA: Enzyme-Linked Immunosorbent Assay
HRP: Horseradish peroxidase
IFN: Interferon
IHC: Immunohistochemistry
IL: Interleukin
LVH: Left Ventricle Hypertrophy
PBS: Phosphate Buffered Saline
PE: Phycoerythrin
SDB: Standard Diluent Buffer
TAC: Transverse Aortic Constriction
TMB: Tetramethylbenzidine
TNF: Tumor Necrosis Factor
1. INTRODUCTION

1.1. HEART FAILURE

Heart failure has become a huge problem in the western world. Every year, approximately 1 million new patients are diagnosed with this illness, which makes it one of the fastest growing cardiovascular diseases. It is mostly common among the elderly. At the age of 40, the prevalence is around 1% and doubles with each decade of life. By the age of 70, the prevalence of heart failure has reached 10%. Although treatment for heart failure has improved in recent years, the outcome is still poor: only 40% has a chance of a 5-year survival (Rodriguez-Artalejo et al., 2004; Devroey & Van Casteren, 2009).

Arnold M. Katz (2001) describes heart failure as 'a clinical syndrome in which heart disease reduces cardiac output, increases venous pressures, and is accompanied by molecular abnormalities which cause progressive deterioration of the failing heart and premature myocardial cell death.'

When the pump function of the heart is defective, blood will back up in the blood vessels, either in the pulmonary artery or in the vena cava superior, depending on the type of heart failure.

In the left ventricular failure, blood will back up in the pulmonary artery, resulting in fluid leaking into the lungs. In the right ventricular failure, blood will accumulate in the vena cava superior, and fluid will leak into the stomach, legs and feet.

The cause of this progressive disease is not yet fully understood. It is known that there is a remodeling of heart tissue that can be a reaction of the heart to a stressful environment. For example in the case of hypertension, the heart has to pump against a higher pressure, and will therefore adapt to this change by increasing in size. This is called hypertrophy, and it is beneficial in the beginning because of the increased force generation, but after reaching its limits the heart will start to fail.

This remodeling is the result of many changes in the heart, such as inflammation, oxidative stress, extracellular matrix remodeling and myocyte stress.
Braunwald (2008) reported that "heart failure results from a complex interplay among genetic, neurohormonal, inflammatory and biochemical changes." This means that the peripheral organs and tissues are involved in the deterioration, and that, as a result, chronic heart failure can be seen as a systemic disease.

The symptoms are in most cases fatigue and shortness of breath. Also edema, lack of appetite and tachycardia are frequently reported. However, patients only suffer from these symptoms in the late stages of the disease. In the beginning, most of them are asymptomatic, which makes it difficult to make an early diagnosis. As a consequence, by the time heart failure is diagnosed, the disease has reached an advanced stage and seems to be irreversible. Thus, in advanced stages of heart failure, treatments are not efficient and can at best only stop, not reverse, the disease process.

Therefore, there is an urgent need for better diagnostic tools, which enable identifying heart failure in early stages and thus permit early therapeutic intervention. Such tools would be of great help to treat heart failure before the quality of life of patients is compromised, and to develop new pharmacotherapeutic options.

Since conventional diagnostic tools have failed so far, biomarkers are believed to facilitate early diagnosis of the disease. Biomarkers are biological substances that give us information about the clinical state of our tissues. Their production is altered as a reaction to certain changes in our body, or they can be the damaging factors themselves. Identification of biomarkers in the early stages of heart failure would open possibilities for earlier diagnoses.

Many different biomarkers for heart failure have already been described, but many of them are solely secreted in the advanced stages, for example the natriuretic peptides. BNP (Brain Natriuretic Peptide), ANP (Atrial Natriuretic Peptide) and CNP (C-type Natriuretic Peptide) are hormones that are produced in response to stretching of the atria or ventricles of the heart. This stretching can be caused by volume or pressure overload. They are nowadays used as biomarkers for heart failure, but since they are secreted rather late in the heart failure process, they are not ideal biomarkers.
The problem with searching for biomarkers is that a huge number of candidate molecules should be screened. However, in the last decade it has become more and more clear that inflammation is an important factor in the progress of this disease and inflammatory mediators are believed to play a key role in the development of heart failure (Anker & Von Haehling, 2004). That is why in our research group it was decided to concentrate on cytokines.

Cytokines are signaling proteins that are involved in the regulation of the immune system, inflammation and in the communication between cells. They circulate in the body in small concentrations and are quickly eliminated. Although one used to think that these inflammatory cytokines were exclusively produced by the immune system, it is now established that they can be synthesized by all nucleated cells in the heart (Mann, 2002). Especially the pro-inflammatory cytokines, such as TNF-\(\text{\textalpha}\) IL-1 and IL-6, and the anti-inflammatory cytokines, such as IL-4 and IL-10, are supposed to be important players in cardiomyopathy.

Our research group is interested to find out if cytokines can be detected in the early stages of heart failure. If so, their profile will be studied: which are the pro-inflammatory and the anti-inflammatory and how do their levels change in time? If they turn out to be good detectable biomarkers, this may pave the way to an earlier diagnosis of heart failure in the future.

1.2. MEASUREMENT OF CYTOKINES

To measure cytokines in a sample, we need an effective method to do so. Immunoassays are nowadays a convenient way. They are based on the specific binding between an antigen and its antibody. Depending on the type of immunoassay, detection is based on the labeling of an antibody or antigen with an enzyme, radioisotope or fluorescence dye. In this work, we will explore the use of ELISA (Enzyme-Linked Immunosorbent Assay) and a multiplex assay.
1.2.1. ELISA

ELISA, or Enzyme-Linked Immunosorbent Assay, is a method for the quantitative or qualitative detection of analytes (in our case the cytokines). This technique is based on the specific binding between an antigen and its antibody, and is therefore highly specific.

We use sandwich-ELISA Kits. The antibodies for the cytokine of interest are coated on each well of a 96-well polystyrene plate. When the serum or plasma is added, the cytokines will strongly interact with their antibodies and form very strong complexes. After washing steps to remove interfering molecules and unbound cytokines, we add secondary antibodies, which will bind to the cytokines and which are conjugated with an enzyme. This enzyme will therefore be indirectly attached to the cytokines. Sometimes, the secondary antibodies are conjugated to a biotin molecule. Biotin has the feature to have a powerful interaction with streptavidin. The streptavidin carries the enzyme responsible for the conversion of the substrate (chromogen) added in the last step, resulting in color development. The intensity of the color is directly related to the amount of cytokines bound to the antibodies, and is measured by the spectrophotometer. This allows us to measure the concentration of the cytokine in the solution. The classical procedure of sandwich-ELISA is illustrated in figure 1.1 below.

The enzyme often used is Horseradish peroxidase (HRP). It catalyzes the oxidation of a substrate by hydrogen peroxide, which leads to a blue color. The most common substrate is tetramethylbenzidine (TMB). To stop this enzymatic reaction, sulfuric acid is added since it denaturates HRP. The color changes from blue to yellow, with a maximum absorption at 450 nm.
FIG. 1.1: ILLUSTRATION OF SANDWICH-ELISA; IN THE FIRST STEP THE ANALYTES BIND TO THEIR ANTIBODIES THAT ARE COATED ON THE WELL. THEN, THE BIOTINYLATED ANTIBODIES ARE ADDED AND BIND TO THE ANALYTES. AFTERWARDS, THE STREPTAVIDIN THAT IS LABELED WITH AN ENZYME CONNECTS TO THE BIOTIN. FINALLY A SUBSTRATE FOR THE ENZYME IS ADDED AND CONVERTED, THEREBY PRODUCING A DETECTABLE COLOR, THAT IS PROPORTIONAL TO THE AMOUNT OF BOUND ANALYTE.

(http://tools.invitrogen.com/content/sfs/manuals/KMC3011_KMC3012%20pr023%20(Ms%20TNF-alpha)%20Rev%201.1.pdf)
1.2.2. Multiplex assay

Although ELISA is a very specific method, it suffers from some disadvantages: only one cytokine at a time can be measured and therefore a lot of sample is needed if one would want to analyze multiple cytokines. Since a few years, new techniques have been developed to overcome these problems, the multiplex assays. They permit to measure multiple analytes in one well, thereby allowing to reduce the amount of sample needed. There can be up to 500 separate tests per well. The principle is the same as in the sandwich-ELISA, but here, the capture antibodies are fixed on a bead in stead of on the well. This is called the xMAP Technology.

Each bead (a polystyrene microsphere) is internally dyed with a different amount of red and infrared fluorophores. By using different intensities of the two dyes, there are 500 sets of microspheres available, each with their own unique signature. This is illustrated in figure 1.2.

FIG. 1.2.: THE BEADS, ON WHICH THE ANTIBODIES ARE FIXED, ARE IMPREGNATED WITH DIFFERENT AMOUNTS OF RED AND INFRARED FLUOROPHORES. THIS PICTURE SHOWS 100 SETS OF BEADS, BUT THESE NOWADAYS CAN BE EXPANDED TILL 500.
(http://www.panomics.com/images/96_2_Bead_v1.jpg)
On these different beads, antibodies, for the cytokines under investigation, are fixed. Each bead carries one type of antibody.

When a sample is brought into a well, which contains all the beads, the cytokines will bind specifically to the right antibodies. After adding the biotinylated detection antibodies and the PE-conjugated streptavidin, PE will be indirectly attached to the cytokines. PE, or Phycoerythrin, is a fluorescence dye that absorbs green light and emits orange light.

The detection, shown in fig. 1.3., is done by the Luminex® 100® Total System. This apparatus sucks up the fluid from the wells which causes the microspheres to line up in a single file. The particles are detected by two lasers, a red and a green one. The red laser is able to detect the color of the bead, and thereby identifies each bead, by exciting the red and infrared dyes. The green laser excites the Phycoerythrin and detects any orange fluorescence that is associated with the binding of the cytokine.

FIG. 1.3.: THE LUMINEX DETECTS THE BEADS AND THE CYTOKINES WITH TWO LASERS. THE RED LASER DETECTS THE COLOR OF THE BEAD, THE GREEN LASER DETECTS THE ORANGE FLUORESCENCE THAT IS ASSOCIATED WITH THE AMOUNT OF CYTOKINES.
http://www.panomics.com/images/96_2_LASER_2_V1.jpg
ELISA and the multiplex assay are therefore based on the same principle, but with a multiplex assay, many cytokines can be measured at a time. However, before we start measuring anything, it is important to assess if what we measure is actually correct. Indeed, many factors have to be taken into account before evaluating measurements. That is why a validation study of the analytical method used is indispensable.

1.3. VALIDATION OF ANALYTICAL METHODS

1.3.1. Reasons for validation

Validations should provide an answer to many questions.
First of all we want to have an idea of the concentration of the cytokines in the sample. Can we measure them at all? How high are the measured values? Will they be below the detection limit or possibly even exceeding the range of the assay? Based on this knowledge the optimal dilution of the sample will be determined in order to be used in the multiplex assays.

Secondly, we want to check if we have to take into account any interferences with our measurements. It is possible that other proteins that are present in our sample could compete with our analyte or the arrayed antibodies. The antigen can also be bound or shielded by proteins, which prevent it from binding its antibody. These effects are collectively called matrix effects.
A matrix effect can be described as the effect that all components of a sample, other than the analyte, have on the measurements. In serum for example, molecules like albumin can interfere with the process. Albumin is typically a carrier protein that could bind the cytokines in the sample, hence preventing them from binding to their antibodies, leading to a lower absorbance.

A next feature to be investigated is the hook effect. The hook effect can be seen in sandwich immunoassays in the presence of two antibodies of which one is labeled. It is a
result of an overwhelming amount of analyte that causes lower absorbance readings than expected (false negative). When there is a huge amount of antigen, the excess can not bind to the labeled antibodies. When the free antigens bind to the coated antibodies on the well, they block them for the antigens that are connected to the labeled antibodies. This causes the absorbance to be lower. The higher the dilution, the lower the concentration of the antigen, and the higher the amount of antigen-antibody complexes that are bound to the coated antibodies. The absorbance measured will then be less aberrant.

The hook effect can also be a result of a too low amount of analyte. If the absorbance is too close to the background, the measurement is not reliable anymore. The concentrations we calculate, without taking into account the dilution factor, will be very close to the detection limit and thus prone to deviation. The error enlarges when multiplying with the dilution factor, and the final concentrations of the higher diluted samples will therefore be much higher than the lower diluted samples, which is impossible.

1.3.2. Dilutions

For the first validation, we measure the absorbance of increasing dilutions of the sample. There are two reasons why we do this.

Firstly, we want to find out which dilution falls in the linear part of our standard curve. This is important to calculate an unknown concentration of cytokines in a sample. If the dilution is too high or too low, the concentration will not be in the linear range, and is therefore not accurate.

Secondly, we want to see if there is a linearity of dilution. This means that if we calculate back our concentration with the dilution factor, we should find the same concentration for all the dilutions.
In some cases, the absorbance reading increases with higher dilutions as a result of the hook effect, as explained in 1.3.1.

1.3.3. Calibration in serum

The standard curve, that we need to interpolate our measurements, is made with reconstituted cytokines (standards) diluted in a standard-diluent buffer. However, the cytokines we want to measure are dissolved in serum or plasma, which have different properties than the standard-buffer. To see if there is a difference between these two solutions, we also make a calibration curve in the sample solution. This is the second validation. By adding a fixed amount of standard concentrations to our sample, we can see how the difference between the buffer and the sample influences the read outs. A difference between the two should be ascribed to a matrix effect, e.g. proteins in the matrix may prevent the antigen from binding to its antibody. On the other hand, the detergent power of the diluent buffer could release the cytokines from the carrier, resulting in a shift of the balance bound/unbound during dilutions.

1.3.4. Standard spikes in serum repetitively diluted with buffer

For the third validation, we spike our sample with a fixed amount of cytokine and repeatedly dilute it in the buffer that we use to dilute our samples and standard solutions. This way we investigate if the buffer has an effect on the measurements. Manufacturers mostly don’t specify the composition of the buffer, but typically they contain detergents that are used to solubilize and stabilize proteins. However, it is possible that they are a disturbing factor. This should be clear if we gradually increase our amount of buffer. That’s why we create a series of dilutions. This effect can therefore also be seen in the dilutions, explained in 1.3.2.

In addition, this enables us to determine the optimal conditions to have a high recovery of the spikes, thus for the analyte.
2. OBJECTIVES

It is clear that heart failure is a serious disease and that the current medication is poorly effective. This is mainly explained by the fact that at the moment the symptoms appear, the heart failure is already in an advanced stage and the damage is irreversible. If heart failure could be detected in an early stage, one would be able to treat it more efficiently and prevent further deterioration.

According to the cytokine hypothesis, cytokines play a major role in the progression of this disease. Therefore cytokines may be promising candidates as biomarkers for heart failure.

We want to see if we can detect and characterize these cytokines in the early stages of heart failure. This research is not very obvious to perform in humans and there are a lot of confounders. It is also uncertain whether the test subjects will indeed develop symptomatic heart failure (unless a retrospective study is performed but in that case frequent sampling is rarely performed). Therefore heart failure is induced in animal models. During the weeks of investigation, we withdraw blood at certain time-points and collect the serum. These samples will then be analyzed for cytokines via biochemical assays, such as ELISA and xMAP multiplexing. After several weeks the animals are sacrificed. The serum is collected and the apex and kidney are preserved to use later on for immunohistochemistry (IHC) investigations.

The goal of this thesis is to validate the ELISA and multiplex assay that are used to measure the cytokines in the samples. This is necessary to check whether our measurements are actually correct. We want to see if there is a matrix effect, a hook effect, or any other interferences that we have to take in account. Also we want to find which dilution is in the linear range of the standard curve. This information should permit to evaluate the results and eventually measure some samples from heart failure animal models. Among the wide list of inflammatory cytokines responsible for heart tissue remodeling, it was decided to first concentrate on the pro-inflammatory cytokines TNF-\(\bar{U}\) and IL-6.
3. MATERIAL AND METHODS

3.1 ANIMAL MODELS

We induce heart failure in animal models, because there are too many confounders in humans and because of ethical aspects. Mice undergo a Transverse Aortic Constriction (TAC) in which we apply a 65-70% constriction between the right and left carotid artery in the aortic arch. In this way, we increase the pressure in the heart, and we induce left ventricular hypertrophy (LVH). After about four weeks, the mice will develop cardiac complications and eventually heart failure (Nakamura et al., 2000).

As control group the Sham mice are used. They are also brought under anesthesia and undergo the same procedure as the TAC-mice, except for the constriction itself. This way, we take into account the stress that is caused by the operation itself.

2, 4 and 6 weeks after the operation, we withdraw around 200 μl of blood and collect the serum. After 6-8 weeks, the mice are sacrificed by bleeding. This time the serum and plasma are collected. In these serum and plasma samples we will measure the cytokines with ELISA and the multiplex assay.

3.2. ELISA

3.2.1 Procedure of validation TNF-α

For the validation of TNF-α, the ELISA Kit for Mouse TNF-α® (Invitrogen™, Camarillo, US) is used. Our sample is serum of TAC-mice.

1. First, a standard curve is established by serial dilutions of a stock solution (5000 pg/ml of TNF-α). These dilutions are made with Standard Diluent Buffer. 400 μl of each standard solution is prepared with a concentration of 1000, 500, 250, 125, 62.5, 31.2 and 15.6 pg/ml TNF-α.
2. For the first validation, the serum sample is diluted. The lower the dilution is, the bigger the chance that we still measure a signal. Therefore we start with a 2-fold dilution, for which 50 μl of serum is added directly to the well. In step 5 we add 50 μl of Standard Diluent Buffer (SDB) to the well, which gives us a 1:1 dilution. For the 4-fold dilution we take 60 μl of the sample and we add 60 μl of SDB (2-fold dilution). From this 120 μl we put 50 μl in the well. After step 5, this will lead to a 4x dilution. If we take 60 μl of this previous 2x-dilution and add 60 μl of SDB, we obtain a 4x-dilution, which leads to an 8x dilution in the well after step 5. The same is done for the 16x dilution, but here we start with the 4x dilution.

3. To investigate possible matrix effects, a calibration curve is also prepared in a fixed amount of serum. We want the final concentration to be the same as the standard solutions. Our fixed amount of serum is 48 μl. To obtain a concentration of 1000 pg/ml, we need to add 12 μl of a 5000 pg/ml standard solution. For the 500 pg/ml, we need to add 12 μl of a 2500 pg/ml standard solution. These standard solutions of 5000, 2500, 1250, 625, 312.5, 156.2, 78.1 and 39.05 pg/ml are made out of the stock solution.

4. To investigate the effect of the buffer on our measurements, we take a fixed amount of serum and standard solution and dilute that in the buffer. With 96 μl of serum we add 24 μl of the 5000 pg/ml stock solution. This way the final concentration is 1000 pg/ml. For the second solution, we take 60 μl of this 1000 pg/ml solution and add 60 μl of buffer, leading to a concentration of 500 pg/ml. We do the same for the following solutions. With each dilution, the effect of the buffer increases.

5. Now that all the solutions are ready, it's time for the ELISA itself. 100 μl of each standard solution is transferred in the correct well. From the diluted serum, the calibration curve in serum and the fixed amount of serum diluted, we take 50 μl, insert it in the correct wells, and add 50 μl of Standard Diluent Buffer. The TNF-Î” in the standard solutions and serum will now bind to the antibodies that are coated on the wells. To see if the buffer itself doesn't give a signal, we add a negative control, which is 100 μl of Standard Diluent Buffer.
6. In the next step, 50 µl of the biotinylated anti-TNF-α is added to each well, except for the negative control. During the incubation of 90 minutes, the biotinylated antibodies will bind to the TNF-α that is bound to the coated antibodies. After this incubation period, the solution is removed and the wells are washed 4 times with a washing buffer to remove unbound antibodies and other components.

7. To each well, except for the negative control, 100 µl of the Streptavidin-HRP solution is added. During the incubation of 30 minutes, the Streptavidin will bind to the Biotin on the antibodies. Therefore, the HRP-enzyme will indirectly be bound to the TNF-α. This is followed again by 4 washing steps to remove the unbound Streptavidin-HRP.

8. Next, 100 µl of the chromogen TMB (tetramethylbenzidine) is added to each well. HRP catalyzes the oxidation of TMB by hydrogen peroxide. The color turns blue and after incubation of 30 minutes, the stop solution will stop the enzymatic reaction and will turn the color yellow.

Using a plate reader the absorbance of each well at 450 nm is determined. After making the fitting curve with the standards, we can calculate the concentration of TNF-α in the serum and see the effect of the serum and the buffer on the measurements.

**3.2.2. Procedure of validation IL-6**

For the validation of IL-6, we use the Quantikine® Mouse IL-6 Immunoassay (R&D Systems, Minneapolis, US). Our serum samples are a pool of 3 wild type mice. In one well we measure the IL-6 in the serum of a TAC mouse obtained from another department.

We prepare standard solutions from the stock solution (500 pg/ml) with a concentration of 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg/ml. The serum of the normal mice is diluted 2, 4 and 8 times for the first validation. For the second validation, the calibration in a fixed amount of serum, we add 30 µl of the 500 pg/ml stock solution with
30 µl of serum to obtain a final concentration of 250 pg/ml. We do the same for the next solutions, always making sure that we have final concentrations that are the same as the standard solutions. For the third validation, we take 60 µl of serum and 60 µl of the 500 pg/ml stock solution. For the next dilutions, we take 60 µl of the previous solution and add 60 µl of the buffer.

To see the amount of IL-6 in TAC-serum, we prepare one well with a 2-fold dilution of the TAC-serum.

The procedure is similar to the one for TNF- in 3.1.2. The difference is that the HRP-enzyme is bound to the anti-IL-6, so there is no specific biotin-streptavidin binding. The substrate for the HRP-enzyme is also TMB.

3.3. MULTIPLEX ASSAY

3.3.1. Procedure of validation Procarta® Cytokine Assay Kit

The kit that is used for the measurement of 9 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, RANTES and TNF-α) is the Procarta® Cytokine Assay Kit (Affymetrix®, Santa Clara, US). Our samples are serum of TAC and SHAM mice, 4 and 6-8 weeks after operation. We also have serum of wild type mice, the controls.

1. The standard solutions are made of a mother solution (20000 pg/ml) with Standard Buffer, with final concentrations of 20000, 5000, 1250, 313, 78, 19.5, 4.9 and 1.22 pg/ml.

2. To see what the absorbance is of the control, we take 20 µl of the control serum and add 60 µl of Standard Buffer (4-fold dilution). We also make dilutions in PBS (Phosphate Buffered Saline). Therefore we take 60 µl of control serum and add 60 µl of PBS (2-fold dilution). For the 4-fold dilution, we take 60 µl of the previous solution and add 60 µl of PBS.
3. Our TAC and SHAM samples are diluted 2 times. We take 30 μl of serum and add 30 μl of PBS.

4. To see if the recovery is good in serum, we add IL-6 standard solutions to a fixed amount of control serum. To receive a final concentration of 250 pg/ml of IL-6, we take 30 μl of serum and add 30 μl of the 500 pg/ml standard solution of IL-6. We do the same for the other solutions, but with decreasing concentrations of IL-6. The final concentrations are 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/ml.

The same procedure is done for TNF-α and the standard solutions, but the final concentrations are 500, 250, 125, 62.5 and 31.2 pg/ml for TNF-α and 10000, 5000, 2500, 1250, 625, 312, 156, 78, 39 and 19 pg/ml for the standard solutions.

This is the second validation.

5. For the third validation, we spike our control serum with an amount of IL-6 and dilute it in buffer. We take 30 μl of serum and add 30 μl of the 500 pg/ml IL-6 solution. From this solution we take 30 μl and we add 30 μl of Assay Diluent. For the next solutions we do the same, therefore having final concentrations of 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.9 pg/ml. Also here we do the same for TNF-α and the standards, with final concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 pg/ml for TNF-α and 5000, 2500, 1250 and 625 pg/ml for the standards.

6. Now that the solutions are ready, the filter plate is pre-wetted with 150 μl of Reading Buffer. After 5 minutes of incubation, the solution is removed with vacuum filtration. Afterwards, 150 μl of the Antibody Beads solution is put into each well, followed by vacuum filtration. Since the beads are too big to go through the filter, they stay in the wells. To wash the beads, 150 μl of washing buffer is added, and then removed with vacuum filtration.

7. 25 μl of Assay Buffer is put into each well, followed by 25 μl of each standard and sample. During the incubation of 60 minutes, the cytokines in the standards and samples will bind to their antibodies on the beads. Afterwards, the wells are washed by removing
the solution with vacuum filtration, and by adding 150 μl of washing buffer to each well. These washing steps are repeated 2 times.

8. 25 μl of the biotinylated antibodies is added to each well in the next step. They will bind to the cytokines that have interacted with their antibodies on the beads. After incubation of 30 minutes, the wells are washed 3 times. Then, 50 μl of the streptavidin-PE conjugate is added and incubated for 30 minutes. The three washing steps are now repeated.

9. In the final step, 120 μl of the Reading Buffer is put into each well. After minimum 5 minutes, the plate can be analyzed by the Luminex®.

3.4. IMMUNOHISTOCHEMISTRY

Besides the immunoassays, there are also alternative methods for the detection of cytokines, for example the visualization in tissue. This is called immunohistochemistry. Immunohistochemistry is a technique that permits to see the distribution of an antigen in tissues. When a tissue is well preserved and labeled antibodies are washed over a slide of this tissue, the antigen-antibody complexes will be visible under a microscope if an enzyme and a substrate are added.

An important step in the procedure of immunohistochemistry is the fixation of the tissue before making the slide. This means that you make sure that the biochemical reactions in the tissue are inhibited, so that it remains in its original state. The most common fixative is formalin, which is a 37% solution of formaldehyde. Formalin creates covalent bonds between proteins. As a result, the proteins aren't soluble anymore and lose their enzymatic functions. Another way to fixate tissue is to freeze it, first in liquid nitrogen and afterwards at -80°C. Each method has its own advantages and disadvantages. In our research group, fixation was done with formalin.
3.4.1. Staining of TNF-\(\alpha\)

After fixation, the tissue is dehydrated in different solutions of alcohol and embedded in paraffin. This block of tissue is then cut into very thin slides of 5 \(\mu\)m thick. At the moment we want to stain them, they are deparaffinized by dipping them in xylol (dimethylbenzene). Since this is a water-insoluble component and the slides need to be washed with water, the slides are hydrated by dipping them in different solutions of ethanol. The enzyme that is used in our research group for the conversion of the substrate is peroxidase. This enzyme is also present in the tissue itself and can interfere with the substrate, leading to incorrect coloring. Therefore a 0.3% solution of hydrogen peroxide is added, to block the endogenous peroxidases.

After a washing-step in 5x PBS (Phosphate Buffered Saline), the antibodies for the analyte in the slide are added, in this case anti-TNF-\(\alpha\). During the incubation period, the antibodies will bind very strongly to the TNF-\(\alpha\) in the slide. The antibodies are produced in goats and are recognized by biotinylated rabbit anti-goat antibodies that are added to the slides in the next step. Just as streptavidin, avidin has the property to interact with biotin. It is a tetrameric protein that can bind four biotin molecules. In the ABC-solution that is added next, avidin is bound to three biotin molecules, which are conjugated with the peroxidase enzyme. The biotin on the antibodies, which are connected to the anti-TNF-\(\alpha\) antibodies, will bind to the free biotin binding spot of avidin. When hydrogen peroxide and diaminobenzidin (DAB) are added, the peroxidase will transform the hydrogen peroxide and DAB will form polymers and precipitate, leaving a brown color.

To have a better view of the cells, the nucleus is also stained with hematoxylin. Finally, the slides are dehydrated by dipping them in solutions of ethanol and xylol, this to preserve the slides and to have a more clear view under the microscope.
3.4.2. Staining of collagen

When the heart experiences stress and becomes hypertrophic, more and more collagen will be produced to maintain the strength of the heart. To see the stage of heart failure in the sacrificed animals, a collagen staining is accomplished in a TAC- and SHAM apex.

The two slides are deparaffinized and hydrated as explained in 3.4.1. To color the background slightly in yellow, they are immersed 5 minutes in Phosphomolybdic acid. The collagen is stained in the next step, when the slides are immersed for 90 minutes in a Sirius Red solution. After this incubation, they are put 2 minutes in 0.01 M HCl and 45 seconds in 70% alcohol before dehydrating them again.
4. RESULTS

4.1. RESULTS OF THE VALIDATIONS

4.1.1. Validation of TNF-Ü

The absorbances of the standard solutions are used to make a calibration curve. First, linear regression is explored. The calibration curve is shown in fig. 4.1. Since the sensitivity of the spectrophotometer decreases above the absorbance of 2, the two highest points should be disregarded. If these two points are removed, a better linear curve is obtained (fig. 4.2).

**FIG. 4.1:** STANDARD CURVE OF TNF-Ü WITH LINEAR REGRESSION.

![ST-curve: linear regression](image)

**FIG. 4.2:** THE SAME STANDARD CURVE AS IN FIG. 4.1. BUT WITH THE TWO HIGHEST OUTLIERS REMOVED. THIS LEADS TO A MORE LINEAR CURVE.

![ST-curve: linear regression (corrected)](image)
However, with this equation in fig. 4.2, negative values were obtained for the concentration of TNF-\(\tilde{\text{U}}\) in the serum sample. Since this is not possible, it is presumed that the results with linear regression are not accurate. That is why a different type of fitting curve is applied. The logarithm of the standard concentration and the absorbances are calculated and a new calibration curve is made. After removing again a few outliers in the highest concentrations, as explained before, the curve in fig. 4.3 is obtained. In contrast to the normal plot (fig. 4.2), the points of measurement now have a more balanced weight on the curve fitting. In fig. 4.2, the high concentration points have a higher weight than the low points.

![St-curve: log](https://via.placeholder.com/150)

**FIG. 4.3: THE FITTING CURVE IS GIVEN BY THE LOGARITHM OF THE ABSORBANCES AND THE STANDARD CONCENTRATIONS.**

With the equation of fig. 4.3, the concentrations of the serum samples, the calibration in serum and the fixed amount diluted in buffer are calculated.

4.1.1.1. First validation: dilutions

The absorbances of the 2x, 4x, 8x and 16x serum dilutions, measured with the ELISA Invitrogen TNF-\(\tilde{\text{U}}\)Kit, are respectively 0.125, 0.084, 0.082 and 0.067 Absorbance Units (AU). It is clear that the absorbance of the 4x dilution is almost half of the 2x, what we would expect in a normal situation. The absorbances of the 8x and 16x dilutions however are not decreasing as they should do. In contrary, they are almost the same as
the 4x dilution. The result is that, when the concentration is calculated with the dilution factor, the concentrations are much higher than the ones in the lower dilutions (2x and 4x). This is illustrated in figure 4.4. The absorbances are also very close to the background (0.06 AU).

![Serum dilutions](image)

**FIG 4.4**: THE CONCENTRATIONS OF TNF-\(\alpha\) IN THE SERUM DILUTIONS CALCULATED WITH THE DILUTION FACTOR.

4.1.1.2. Second validation: calibration in serum

The concentration of TNF-\(\bar{U}\) in serum, to which a known amount of TNF-\(\bar{U}\) was added, is calculated. The concentration that is expected, the one that is observed and the recovery are illustrated in table 4.1.

It is clear that the recovery of TNF-\(\bar{U}\) in serum is very low. To see if this decrease in absorbance in serum is a constant factor, the observed TNF-\(\bar{U}\) concentration in buffer is plotted against the one in serum. This curve is shown in figure 4.5. The observed concentrations in buffer and serum are the concentrations that are calculated with the equation in fig. 4.3.
TABLE 4.1: THE EXPECTED AND OBSERVED CONCENTRATIONS AND THE RECOVERY OF TNF-α IN A FIXED AMOUNT OF SERUM TO WHICH WE ADDED A KNOWN AMOUNT OF TNF-α.

<table>
<thead>
<tr>
<th>Expected concentration (pg/ml)</th>
<th>Observed concentration in serum (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>315.11</td>
<td>31.5</td>
</tr>
<tr>
<td>500</td>
<td>209.53</td>
<td>41.9</td>
</tr>
<tr>
<td>250</td>
<td>113.67</td>
<td>45.5</td>
</tr>
<tr>
<td>125</td>
<td>68.73</td>
<td>55.0</td>
</tr>
<tr>
<td>62.5</td>
<td>35.22</td>
<td>56.4</td>
</tr>
<tr>
<td>31.3</td>
<td>21.88</td>
<td>70.0</td>
</tr>
<tr>
<td>15.6</td>
<td>11.83</td>
<td>75.7</td>
</tr>
</tbody>
</table>

FIG. 4.5: THE OBSERVED STANDARD CONCENTRATION OF TNF-α IN BUFFER PLOTTED AGAINST THE OBSERVED CONCENTRATION IN SERUM. THEY ARE CALCULATED WITH THE EQUATION IN FIG. 4.3.
4.1.1.3. Third validation: standard spike in serum diluted with buffer

To see the effect of the buffer, serum was spiked with a standard concentration and repeatedly diluted in buffer. The expected and observed concentration and the recovery are shown in table 4.2.

TABLE 4.2: THE EXPECTED AND OBSERVED CONCENTRATION AND THE RECOVERY OF TNF-α. A CONCENTRATION OF 5000 PG/ML WAS SPIKED IN SERUM AND REPEATEDLY DILUTED IN BUFFER.

<table>
<thead>
<tr>
<th>Expected concentration (pg/ml)</th>
<th>Observed concentration (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>310.39</td>
<td>31.0</td>
</tr>
<tr>
<td>500</td>
<td>214.17</td>
<td>42.8</td>
</tr>
<tr>
<td>250</td>
<td>126.99</td>
<td>50.8</td>
</tr>
<tr>
<td>125</td>
<td>64.87</td>
<td>51.9</td>
</tr>
<tr>
<td>62.5</td>
<td>32.26</td>
<td>51.6</td>
</tr>
<tr>
<td>31.3</td>
<td>17.02</td>
<td>54.5</td>
</tr>
<tr>
<td>15.6</td>
<td>9.58</td>
<td>61.3</td>
</tr>
</tbody>
</table>

4.1.2. Validation of IL-6

The standard curve of IL-6 is obtained by plotting the logarithm of the standard concentrations versus the logarithm of the absorbances.

4.1.2.1. First validation: dilutions

The samples, measured with the ELISA R&D Systems™ IL-6 Kit, are diluted 2, 4 and 8 times. The results of these dilutions are shown in table 4.3. With the equation of the standard curve, the concentration of IL-6 in the dilutions is calculated. This is the observed concentration. However, table 4.4 shows that the recovery in serum is not 100%. The decrease in recovery is linear though, so the real concentration can be calculated
back with the equation in fig. 4.6 after calculating the corrected concentration with the
dilution factor.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Absorbance (AU)</th>
<th>Abs (^{-1}) blank (AU)</th>
<th>Observed conc. (pg/ml)</th>
<th>Corrected conc. (pg/ml)</th>
<th>Real conc. (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x</td>
<td>0.087</td>
<td>0.013</td>
<td>4.07</td>
<td>4.07</td>
<td>8.93</td>
</tr>
<tr>
<td>4x</td>
<td>0.086</td>
<td>0.012</td>
<td>3.77</td>
<td>7.54</td>
<td>12.59</td>
</tr>
<tr>
<td>8x</td>
<td>0.094</td>
<td>0.020</td>
<td>5.97</td>
<td>23.87</td>
<td>29.80</td>
</tr>
</tbody>
</table>

4.1.2.2. Second validation: calibration in serum

The results of the second validation are shown in table 4.4. The range of recovery that is
accepted in our research group is 80-120%. To check if the change in serum is linear, the
observed concentration in serum is plotted against the observed concentration in buffer
(fig. 4.6.).

<table>
<thead>
<tr>
<th>Expected conc. (pg/ml)</th>
<th>Observed conc. in serum (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>243.71</td>
<td>97.5</td>
</tr>
<tr>
<td>125</td>
<td>113.07</td>
<td>90.5</td>
</tr>
<tr>
<td>62.5</td>
<td>50.91</td>
<td>81.5</td>
</tr>
<tr>
<td>31.25</td>
<td>29.87</td>
<td>95.7</td>
</tr>
<tr>
<td>15.625</td>
<td>20.53</td>
<td>131.6</td>
</tr>
<tr>
<td>7.8</td>
<td>12.05</td>
<td>154.5</td>
</tr>
<tr>
<td>3.9</td>
<td>15.91</td>
<td>408.0</td>
</tr>
</tbody>
</table>
4.1.2.3. Third validation: standard spike in serum diluted with buffer

The expected and observed concentration and the recovery of IL-6 in serum, spiked with a standard concentration and diluted with buffer, are shown in table 4.5.

TABLE 4.5: THE EXPECTED AND OBSERVED CONCENTRATION AND RECOVERY OF IL-6. A CONCENTRATION OF 500 PG/ML WAS SPIKED IN SERUM AND REPEATEDLY DILUTED IN BUFFER.

<table>
<thead>
<tr>
<th>Expected conc. (pg/ml)</th>
<th>Observed conc. (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>203.09</td>
<td>81.2</td>
</tr>
<tr>
<td>125</td>
<td>115.10</td>
<td>92.1</td>
</tr>
<tr>
<td>62.5</td>
<td>52.39</td>
<td>83.8</td>
</tr>
<tr>
<td>31.25</td>
<td>29.71</td>
<td>95.2</td>
</tr>
<tr>
<td>15.625</td>
<td>14.78</td>
<td>94.7</td>
</tr>
<tr>
<td>7.8</td>
<td>9.47</td>
<td>121.4</td>
</tr>
<tr>
<td>3.9</td>
<td>6.62</td>
<td>169.9</td>
</tr>
</tbody>
</table>
4.1.2.4. TAC-sample

The observed and real concentration of IL-6 in the TAC-sample are illustrated in table 4.6. The observed concentration is calculated with the equation of the standard curve in buffer. The real concentration is calculated with the equation in fig. 4.6.

<table>
<thead>
<tr>
<th>Absorbance - blank</th>
<th>Observed conc. (pg/ml)</th>
<th>Real conc. (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.272</td>
<td>73.05</td>
<td>81.64</td>
</tr>
</tbody>
</table>

**TABLE 4.6: THE OBSERVED AND REAL CONCENTRATION OF IL-6 IN THE TAC SAMPLE**

4.1.3. Validation of the multiplex assay

4.1.3.1. Standard solutions

The observed standards concentrations are calculated and illustrated in fig. 4.7. These values are compared to the expected ones. As an example, the observed and expected standards concentrations of IL-2 are shown in fig. 4.8. These results are similar for the other 8 cytokines.

4.1.3.2. Standards in serum

To see if there is a matrix effect, the standard solutions were added to control serum. There are different results for the cytokines. The expected and observed concentration and recovery of IL-1β are shown in table 4.7. The recovery is very low and appears to decrease with lower concentrations of IL-1β. Similar results are obtained for IL-4, IL-6 and IL-10. In the recovery of IL-1β and IL-2 however, no trend can be observed. Table 4.8 and figure 4.9 show the results of IL-1β as an example. Interferon-γ, RANTES and TNF-α all have such a low recovery, that only the highest concentrations are detected. Table 4.9 and figure 4.10 show the results for TNF-α.
FIG. 4.7: THE EXPECTED AND OBSERVED STANDARD CONCENTRATIONS OF THE 9 CYTOKINES, MEASURED WITH THE MULTIPLEX ASSAY.

FIG. 4.8: THE EXPECTED AND OBSERVED STANDARD CONCENTRATIONS OF IL-2.
### TABLE 4.7: THE EXPECTED AND OBSERVED CONCENTRATION AND RECOVERY OF IL-1 α IN CONTROL SERUM

<table>
<thead>
<tr>
<th>Expected conc. (pg/ml)</th>
<th>Observed conc. (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>3119.3</td>
<td>31.2</td>
</tr>
<tr>
<td>5000</td>
<td>1762.4</td>
<td>35.3</td>
</tr>
<tr>
<td>2500</td>
<td>629.1</td>
<td>25.2</td>
</tr>
<tr>
<td>1250</td>
<td>228.7</td>
<td>18.3</td>
</tr>
<tr>
<td>625</td>
<td>105.9</td>
<td>16.9</td>
</tr>
<tr>
<td>312.5</td>
<td>37.8</td>
<td>12.1</td>
</tr>
<tr>
<td>156.3</td>
<td>18.0</td>
<td>11.5</td>
</tr>
<tr>
<td>78.1</td>
<td>7.4</td>
<td>9.2</td>
</tr>
<tr>
<td>39.1</td>
<td>&lt; detection limit</td>
<td>-</td>
</tr>
</tbody>
</table>

### TABLE 4.8: THE EXPECTED AND OBSERVED CONCENTRATION AND RECOVERY OF IL-1β IN CONTROL SERUM.

<table>
<thead>
<tr>
<th>Expected conc. (pg/ml)</th>
<th>Observed conc. (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>4226.8</td>
<td>42.3</td>
</tr>
<tr>
<td>5000</td>
<td>2560.5</td>
<td>51.2</td>
</tr>
<tr>
<td>2500</td>
<td>1234.5</td>
<td>49.4</td>
</tr>
<tr>
<td>1250</td>
<td>471.0</td>
<td>37.7</td>
</tr>
<tr>
<td>625</td>
<td>264.4</td>
<td>42.3</td>
</tr>
<tr>
<td>312.5</td>
<td>96.3</td>
<td>30.9</td>
</tr>
<tr>
<td>156.3</td>
<td>52.6</td>
<td>33.7</td>
</tr>
<tr>
<td>78.1</td>
<td>43.2</td>
<td>55.3</td>
</tr>
<tr>
<td>39.1</td>
<td>30.8</td>
<td>79.1</td>
</tr>
<tr>
<td>19.6</td>
<td>&lt; detection limit</td>
<td>-</td>
</tr>
</tbody>
</table>
FIG. 4.9: THE OBSERVED AND EXPECTED CONCENTRATION OF IL-1β IN BUFFER AND THE OBSERVED CONCENTRATION IN SERUM.

TABLE 4.9: THE EXPECTED AND OBSERVED CONCENTRATION AND RECOVERY OF TNF-α IN CONTROL SERUM.

<table>
<thead>
<tr>
<th>Expected conc. (pg/ml)</th>
<th>Observed conc. (pg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000</td>
<td>1936.5</td>
<td>19.4</td>
</tr>
<tr>
<td>5000</td>
<td>1477.5</td>
<td>29.5</td>
</tr>
<tr>
<td>2500</td>
<td>515.4</td>
<td>20.6</td>
</tr>
<tr>
<td>1250</td>
<td>227.0</td>
<td>18.2</td>
</tr>
<tr>
<td>625</td>
<td>113.0</td>
<td>18.1</td>
</tr>
<tr>
<td>312.5</td>
<td>&lt; detection limit</td>
<td>-</td>
</tr>
</tbody>
</table>

FIG. 4.10: THE OBSERVED AND EXPECTED CONCENTRATION OF TNF-α IN BUFFER AND THE OBSERVED CONCENTRATION IN SERUM.
4.1.3.4. Samples

A lot of TAC- and SHAM samples are measured and the results are shown in table 4.12. Our research group is especially interested in the amount of IL-6 that is measured in the same sample (TAC 9) that was used in the validation of ELISA IL-6. With the ELISA Kit, an IL-6 amount of 81 pg/ml was calculated (table 4.6). The observed concentration of IL-6 in the multiplex assay is 13.8 pg/ml. The real concentration is illustrated in table 4.13. IL-1β, IL-4, IFN-γ, RANTES and TNF-α were not detected in any sample and therefore not illustrated in table 4.12.

TABLE 4.12: CONCENTRATIONS (PG/ML) OF IL-1β, IL-2, IL-6 AND IL-10 MEASURED IN DIFFERENT TAC- AND SHAM-SAMPLES.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAC 1</td>
<td>80.8</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>TAC 2</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
<td>-</td>
</tr>
<tr>
<td>TAC 3</td>
<td>9.4</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAC 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAC 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAC 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAC 7</td>
<td>80.5</td>
<td>11.2</td>
<td>9.1</td>
<td>18.5</td>
</tr>
<tr>
<td>TAC 8</td>
<td>30.5</td>
<td>8.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAC 9</td>
<td>25.5</td>
<td>7.8</td>
<td><strong>13.8</strong></td>
<td>-</td>
</tr>
<tr>
<td>TAC 10</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>SHAM 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SHAM 2</td>
<td>13.6</td>
<td>3.5</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>SHAM 3</td>
<td>7.7</td>
<td>3.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SHAM 4</td>
<td>126.9</td>
<td>14.9</td>
<td>10.4</td>
<td>35.1</td>
</tr>
<tr>
<td>SHAM 5</td>
<td>-</td>
<td>-</td>
<td>10.1</td>
<td>-</td>
</tr>
<tr>
<td>CONTROL</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE 4.13: MEASUREMENTS OF THE IL-6 CONCENTRATION IN SAMPLE TAC 9 WITH THE ELISA AND THE MULTIPLEX ASSAY. THE REAL CONC. IS THE OBSERVED CONC. BUT CORRECTED FOR THE EFFECT IN SERUM.

<table>
<thead>
<tr>
<th></th>
<th>Observed conc. (pg/ml)</th>
<th>Real conc. (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>73.1</td>
<td>81.6</td>
</tr>
<tr>
<td>Multiplex assay</td>
<td>13.8</td>
<td>38.3</td>
</tr>
</tbody>
</table>
4.2. RESULTS OF IMMUNOHISTOCHEMISTRY

The results of the staining of TNF-α in the apex of TAC- and SHAM-mice are shown in fig. 4.9 and 4.10. TNF-α is colored in dark brown.

FIG. 4.9: APEX OF TAC-MOUSE WITH TNF-α STAINED.

FIG. 4.10: APEX OF SHAM-MOUSE WITH TNF-α STAINED.

To see the evolution of heart failure, a collagen staining of the apex was also accomplished. These results are shown in fig. 4.11 and 4.12. Collagen is colored in red.
FIG. 4.11: COLLAGEN STAINING IN APEX OF TAC-MOUSE (40X)

FIG. 4.12: COLLAGEN STAINING IN APEX OF SHAM-MOUSE (40X)
5. DISCUSSION

5.1 ELISA: TNF-ẞ

5.1.1. First validation: dilutions

In fig. 4.4 it is shown that the calculated concentrations of the 8- and 16-fold dilutions are much higher than the 2- and 4-fold dilutions. This is referred to as the hook effect. The absorbances of the 4-, 8- and 16-fold dilutions are quite similar. Therefore if the concentrations are calculated with the dilution factors, the higher dilutions have higher concentrations, which is impossible.

There can be two explanations for this observation.

First of all, the measurements are too close to the background (0.06 AU). The background is the absorbance of the solution in which our standards and samples are diluted, in this case the Standard Diluent Buffer. To have accurate results, it is recommended that the absorbances of the samples are twice the background. The 2-fold dilution of our serum sample has an absorbance of 0.125 AU, which is more than twice the background and therefore acceptable according to this rule. The other dilutions however have absorbances of 0.084, 0.082 and 0.067 AU, which are too close to the background and therefore not reliable.

So the only dilution at which we can rely on is the 2-fold dilution, in which we find a concentration of 19 pg/ml of TNF-ẞ.

A second explanation can be that certain proteins, such as carriers, in the serum bind to TNF-ẞ and therefore inhibit the interaction with the antibodies. The detergent power of the buffer however can release the TNF-ẞ from these proteins, therefore increasing the amount that can bind to the antibodies. However, this will reach a maximum, because further dilution of the sample will eventually lead to a decrease of the absolute amount of antigen available for binding. Since this is not observed (as the 8- and 16-fold dilutions do not differ greatly from the 4-fold dilution), the first explanation seems more probable.
Another reason to do this validation was to find which dilutions are in the linear range of the standard curve. However, fig. 4.3 shows that we have a linear curve, albeit only in the low range, and that our concentrations are interpolated by the curve, so this question is irrelevant.

In literature, there are not many data available of TNF-α levels, especially not in mice. A similar study of Lachtermacher et al. (2010) to investigate the cytokine profile in mice with heart failure showed a TNF-α concentration of 1000 pg/ml, which is significantly higher than our measurement of 19 pg/ml. However, they used a multiplex cytokine kit of Bio-Rad, which our research group also used in previous experiments with rats and with which extremely high measurements were obtained. The dilutions didn’t show linearity but mainly hook effects. The Bio-Rad ELISA Kit for mice had a little better results, but they were still too high to be reliable. Because of this, the results of Lachtermacher et al. are quite questionable, and this proves that there is a growing need for validations.

Also Vistnes et al. (2010), who measured circulating cytokine levels in mice with heart failure, found TNF-α concentrations of more than 1000 pg/ml, but again the Bio-Rad multiplex cytokine kit was used and no validations were accomplished.

Another study of Cesari et al. (2003) in humans who were developing heart failure, showed a TNF-α level of 3.4 pg/ml. ELISA kits for human samples are generally more validated and more sensitive than kits for animals, so these data can be assumed to be more representative than the data in animals.

It is clear that the little data that are available are not consistent, and that more research is necessary.

Overall, the TNF-α levels are supposed to be very low. Since the half-life is very short, a future solution can be to measure TNF-α levels in urine.
5.1.2. Second validation: calibration in serum

In table 4.1, it is shown that by adding standard solutions of TNF-\(\tilde{\alpha}\) to serum instead of buffer, the recovery decreases significantly. It is remarkable that the recovery of high concentrations is very low, but increases when the concentration decreases. Normally, it would be expected that the recovery stays the same or decreases with lower concentrations. That is because there can be soluble TNF-\(\tilde{\alpha}\) receptors in the serum that can bind TNF-\(\tilde{\alpha}\), therefore inhibiting the interaction with the antibodies. In low concentrations, all the TNF-\(\tilde{\alpha}\) molecules can be bound by the receptors, but when the concentration is higher, it is possible that the receptors are saturated, and therefore the free TNF-\(\tilde{\alpha}\) concentration is higher.

However, in our measurements the opposite effect is seen. An explanation for the fact that the recovery is lower with high concentrations can be that the TNF-\(\tilde{\alpha}\) molecules stick together and form oligomers. Therefore the antibodies cannot detect all the TNF-\(\tilde{\alpha}\) molecules and the recovery is lower. In lower concentrations, this tendency to form oligomers is less likely.

Even in the low concentrations, the recovery is still not in the acceptable range (80-120\%). That is why it is supposed that TNF-\(\tilde{\alpha}\) is not a very stable molecule.

To see if this decrease in recovery is constant, the observed concentration in serum was plotted against the observed concentration in buffer. Fig. 4.5. shows clearly that there is a linear relationship between the recovery in serum and the real recovery in buffer. This fact is advantageous, because with this equation, the real concentration can be calculated.

For future measurements, it seems necessary to do this validation each time, maybe not with all the standard solutions, but with a high, medium and a low concentration.
5.1.3. Third validation: standard spike repeatedly diluted with buffer

In table 4.2, the effect of the buffer on the recovery can be seen. Similar to the second validation, the recovery of the high concentrations is very low. The more the samples are diluted with buffer, the higher it gets. This can be due to the clustering of the TNF-\( \alpha \) molecules that decreases with lower concentrations. However, the recovery is lower than in the second validation. This suggests that TNF-\( \alpha \) is less stable, i.e. degraded more rapidly, in buffer than in serum. Manufacturers do not include the composition of their buffer in their kits, but buffers always contain detergents that could interact with TNF-\( \alpha \). These can have a negative effect on the recovery. Alternatively, it is possible that serum provides protection for TNF-\( \alpha \) against oxidation or other (bio)chemical conversion reactions.

5.2. ELISA: IL-6

5.2.1. First validation: dilutions

Similar to TNF-\( \alpha \), there is no linearity in the dilutions of the samples. The 2-, 4- and 8-fold dilutions have almost the same absorbance, which means that the concentrations increase with higher dilutions. This can again be explained by the fact that the values are too close to the background, which is 0.074 AU. The amount of IL-6 in the control serum is therefore under the detection limit.

5.2.2. Second validation: calibration in serum

Unlike the low recovery of TNF-\( \alpha \), the recovery of IL-6 in high concentrations is definitely in the acceptable range (table 4.4). Apparently, IL-6 does not have the tendency to cluster. The fact that the recovery in lower concentrations increases can be because the absorbance is getting close to the background. The absorbance of the 15.6 pg/ml solution
(0.146 AU) is still more than twice the background (0.074 AU), but in the 7.8 pg/ml solution (0.129 AU), that is not the case anymore, and the recovery increases to 155%. The absorbance of the 3.9 pg/ml solution is 0.115 AU, which is so close to the background that the recovery is over 400%, and clearly not reliable anymore.

5.2.3. Third validation: standards spike repeatedly diluted with buffer

Also in this validation, the recovery is quite acceptable (table 4.5) but lower than in serum. Again, this can be explained by detergents in the buffer or by protection by the serum against degradation. The recovery in the two lowest concentrations increases above 120%, but this is again due to the background.

5.2.4. TAC-sample

The absorbance of the TAC-sample is 0.272 AU, which is high above the background. Since the decrease in recovery in serum is constant, it is possible to calculate the real concentration with the equation found in fig. 4.6. A result of 81 pg/ml is obtained.

Vistnes et al. (2010) analyzed IL-6 with a validated ELISA method of R&D Systems in different heart failure mice models and found levels between 40 and 140 pg/ml. This corroborates with our measurement. Since the amount of IL-6 in controls is negligible, it does seem that IL-6 is produced in heart failure. However, it is possible that the IL-6 level depends on other cytokines as well. In the end, it is our goal to find patterns between different cytokines, but IL-6 could be a possible biomarker for heart failure in the future.
5.3 MULTIPLEX ASSAY

5.3.1. Standard solutions

In fig. 4.8, it is clear that the expected and observed concentration of IL-2 correlate very well. This is also noticed with the other cytokines, which means that the accuracy of the Luminex is high. On the other hand, fig. 4.7 shows that low concentrations do not lie in the linear range of the standard curves. For IL-4, the linear range starts around 50 pg/ml, for IL-1α IL-2, IL-6, IGN-γ and RANTES it starts around 100 pg/ml and for TNF-IL-1β and IL-10 only around 500 pg/ml. Measurements need to lie in the linear range to be reliable, therefore samples with low amounts of cytokines are not really useful.

5.3.2. Standards in serum

Since the recovery of each cytokine is so low in serum, there is an obvious matrix effect. For IL-1α, IL-4, IL-6 and IL-10, the recovery decreases as the concentrations decrease. An explanation for this phenomenon could be that cytokines in low concentrations don’t bind to the beads and that maybe a longer incubation time is necessary. However, the standard solutions are almost perfectly detected by the Luminex (5.3.1), even the solutions with low concentrations. Therefore, the low recovery is definitely due to a matrix effect. Probably, serum factors decrease the ability of the cytokines to bind to the beads.

It is possible that there are soluble receptors for the cytokines in the serum that bind the cytokines, which makes it impossible for them to bind to the antibodies on the beads. As a result the recovery decreases. However, the higher the concentration of the cytokine, the more saturated the receptors are, and the higher the recovery is.
For IL-1β and IL-2, a trend in recovery can not be observed. The recovery varies between 30 and 50% for both cytokines. Nonetheless, the recovery of IL-1β in the two lowest concentrations increases, but this can be due to the background.

Interferon-γ, RANTES and TNF-α have each such a low recovery, that only the highest concentrations can be detected. From previous experiments with ELISA, it is already known that TNF-α is not a stable molecule and that it tends to cluster in high concentrations (5.1.2). When table 4.1, the recovery of TNF-α in serum with ELISA, and table 4.9, the recovery of TNF-α in serum with the multiplex assay, are compared, it is clear that lower concentrations can still be detected with ELISA and not with the multiplex. Figures 4.9 and 4.10 show the recovery of IL-1β and TNF-α in serum. In these pictures it is clear that low concentrations are not detectable. Because of these big variabilities in recovery between cytokines, it is necessary to do validations with each measurement.

5.3.4. Samples

To compare ELISA with the multiplex assay, the same sample in which we measured IL-6 with the ELISA Kit is used to determine IL-6 with the multiplex. The results are shown in table 4.13. It is clear that the amount measured with the multiplex is a lot less than the amount measured with ELISA. This proves again that the sensitivity of the multiplex is lower. The linear range of the standard curve of IL-6 in the multiplex starts around 100 pg/ml, so the determined concentration is not reliable. This is also observed for the other cytokines that are determined in different samples (table 4.12). If there are any cytokines, their concentration is too low to be trustworthy.

There is one sample, SHAM 4, in which multiple cytokines are measured. Since this is a SHAM-mouse and not a mouse with possible heart disease and since there are no cytokines found in other SHAM-samples, this result can be seen as an outlier.
Since the sensitivity of the multiplex is so low, it is hard to make conclusions based on these results. It is possible that there is a cytokine pattern in mice with cardiac disease, but to confirm this, a more sensitive method should be used.

5.4 IMMUNOHISTOCHEMISTRY

The results of the TNF-Üstaining in the apex are shown in fig. 4.7 and 4.8. Since there is a lot of background staining, it is quite difficult to distinguish the places with TNF-Ü from the artifacts. What can be seen is that, in both TAC- and SHAM, TNF-Ü is present in the capillaries. There is not much known in literature about the expression of TNF-Ü in the heart, so this observation cannot be compared with other findings. It is known that, in the presence of inflammation, the capillary permeability increases and that different leucocytes are attracted that produce cytokines. However, there is no significant difference between TAC and SHAM. This can have different reasons.

Firstly, it is possible that there was, by coincidence, an inflammation process going on in the SHAM mouse. Secondly, it can be that the stage of heart failure is too early to see a real inflammation. This can be examined with the collagen staining. The pictures of the collagen staining (fig. 4.11 and 4.12) show that there is a difference between TAC and SHAM. The TAC-slide clearly shows more collagen. Collagen is produced when the muscle tissue expands, this to give the heart more strength. An excessive deposition of collagen is responsible for stiffness of the heart, and is important in the remodeling process of heart failure. Seeing more collagen in the TAC than in SHAM means that the heart already started to become hypertrophic. Therefore it can be assumed that heart failure is at least in its early stages.

Since there is no clear difference in TNF-Üstaining, it can be that TNF-Ü is not the main cytokine to look for in heart failure, given that there were also no significant values found in serum. For the future, it would be useful to look at the distribution of other cytokines, especially IL-6, since that cytokine has proven to be increased in serum.
6. CONCLUSIONS

A lot of differences and similarities are seen between cytokines while evaluating the validations. TNF-\(\alpha\) and IL-6 were both measured in serum samples with ELISA kits from different manufacturers. For both cytokines, a clear hook effect was observed. The concentration in the samples were very low, and the absorbances of the dilutions were therefore too close to the background and not reliable anymore. Only in the 2-fold dilution of TNF-\(\alpha\) a reliable amount of 19 pg/ml could be identified. No reliable amount of IL-6 was found in control serum, but in a TAC-sample, a concentration of 81 pg/ml was measured. The problem with low amounts is that it is difficult to obtain reliable results because of the background. Therefore it is important to have a high accuracy. Unfortunately, the sensitivity that is proposed by manufacturers is most of the time not met.

Another resemblance is the effect of the buffer on the measurements. The recovery of TNF-\(\alpha\) and IL-6 was lower in serum after diluting with buffer, which is most probably due to the presence of detergents in the buffer, that interact with the cytokines.

A difference between TNF-\(\alpha\) and IL-6 is the recovery in serum. The recovery of TNF-\(\alpha\) is very low and apparently it tends to form oligomers in high concentration. This trend to cluster diminishes as the concentration decreases. As a result, the recovery of TNF-\(\alpha\) increases as the concentration decreases. Since this is also observed in buffer, clustering of TNF-\(\alpha\) is cannot be seen as a matrix effect.

IL-6 has not the feature to cluster in high concentration, and the recovery in serum is in the acceptable range, between 80 and 120%.

A multiplex assay was also used to determine the amount of 9 cytokines (IL-1\(\alpha\), IL-1\(\beta\), IL-2, IL-4, IL-6, IL-10, IFN-\(\gamma\), RANTES and TNF-\(\gamma\)) in different samples. Overall, the assay showed a low sensitivity and is therefore not suitable for the measurement of low amounts.

A comparison was made between ELISA and the multiplex assay by measuring IL-6 in the same sample. In the ELISA kit, an amount of 81 pg/ml was measured, in the
multiplex assay 38 pg/ml. An explanation for the this difference is that, in the multiplex assay, the linear range of the standard curve starts only around 100 pg/ml, which makes this result unreliable. The ELISA seems therefore more appropriate.

The recovery in serum of these 9 cytokines was also examined. The results differ for each cytokine but was generally very low. Because there is no common trend, it is necessary to do this validation for each measurement and for each method.

The results of the TNF-α staining of the apex showed no significant difference between TAC and SHAM. To see if there was in fact heart failure developing, a collagen staining was performed, which showed that the TAC had more collagen and was therefore in the hypertrophic stage. It is possible that TNF-α is not a good biomarker for heart failure. Therefore it would be useful to do an IL-6 staining, because IL-6 showed a more significant increase in serum than TNF-α.

On first sight, these results do not suggest that cytokines are detectable in the early stages of heart failure. The amounts that are determined in the samples are low and raise the question if cytokines can be good biomarkers in the future. Nonetheless, it is possible that with other, more sensitive, methods cytokine patterns can be discovered. After all, with the multiplex assay only 9 cytokines were measured. There are still a lot of other cytokines that can be investigated. Whatever method that will be used in the future, it is necessary to do validations with each measurement to discover hook effects, matrix effects or other disturbing factors that confound the results. Otherwise it is doubtful that accurate results are obtained.
7. LITERATURE


http://www.panomics.com/images/96_2_Bead_v1.jpg

http://www.panomics.com/images/96_2_LASER_2_V1.jpg

http://tools.invitrogen.com/content/sfs/manuals/KMC3011_KMC3012%20pr023%20(Ms%20TNF-alpha)%20Rev%201.1.pdf


