**Diplomarbeit**

# Comprehensive Liquid Biopsy in Patients after Organ Transplant

## cfDNA/microRNA and immune cell repertoire as markers for tissue damage and organ rejection


eingereicht von

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# Preface

*"Fall in love with some activity and do it! Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough. Work as hard and as much as you want to on the things you like to do the best. Don't think about what you want to be, but what you want to do. Keep up some kind of a minimum with other things so that society doesn't stop you from doing anything at all." Richard P. Feynman*

# Zusammenfassung


## Ziele

Ziel dieser Pilotstudie ist es die Möglichkeiten der “Liquid Biopsy” als Biomarker der Transplantatabstoßung auszuloten und die Methodik dafür im Bereich der Diagnostik zu etablieren. Eine Abstoßungsreaktion präsentiert sich klinisch als Gewebsschaden. Dieser Schaden ist bedingt durch die immunologische Reaktion auf das Spenderorgan, als auch durch Ischämieschäden. Aufgrund des Zelltods werden Nukleinsäuren des Fremdorgans in den Blutstrom entlassen. Diese vom Spenderorgan stammende zellfreie DNA sowie zellfreie microRNA kann dazu verwendet werden eine - sowie auch die Schwere - der Abstoßungsreaktion als minimal-invasive Methode festzustellen. Weiteres wird die Immunreaktion selbst anhand der T-Zellrezeptor Diversität aufgezeigt, welche sich durch die klonale T-Zell-Expansion bei Immunreaktion verändert.

## Methoden

### Studienkohorte und Zeitplan

Von August 2018 bis August 2020 findet eine prospektive Sammelstudie der Medizinischen Universität Graz statt. In dieser Studie werden voraussichtlich 200 Personen eingeschlossen werden, welche mittels Organtransplantation (Leber, Nieren) behandelt werden. Blutproben werden zu den Zeitpunkten Tag 0, Tag 7-10, 1 Monat (+/- 1 Woche), 1 Jahr (+/- 1 Monat) abgenommen sowie zu dem optionalen Ereignis: Verdacht auf Transplantatabstoßung.

### Isolierung von Nukleinsäure & Leukozyten

Zellfreie DNA wird mittels QIAamp Circulating Nucleic Acid Kit® (Qiagen, Hilden, Germany) isoliert. Anschließend wird die eine SNP-Analyse mittels HID-Ion AmpliSeq Identity Panel® durchgeführt. MicroRNA wird mit dem miRNeasy Plasma/Serum Advanced Kit® (Qiagen, Hilden, Germany) aufbereitet. Die DNA der mononukleären Zellen wird nach Isolation für eine Multiplex-PCR der CDR3 Region verwendet (1).

## Resultate & Zusammenfassung

Eine Sammelstudie wurde etabliert und erste Pilotproben der Studie bereits analysiert, und anhand derer wurden die Labormethoden zur Analyse von DNA, microRNA sowie T-Zell Rezeptorvariabilität etabliert und überprüft. Die zellfreie DNA kann bis zu einer Nachweißgrenze von 0,1 % Spender-DNA im Blut diskriminiert werden. Das microRNA-Profil sowie die T-Zell Diversität können ebenfalls auf eine Abstoßungsreaktion hindeuten und erweisen sich als robust. Die Studie hat die Zustimmung der Ethikkommission der Medizinische Universität Graz. Weiteres werden aufbauend auf diese Pilotstudie, mindestens 160 Personen innerhalb eines Beobachtungszeitraumes von 2 Jahren nach Transplantation (Leber, Niere) eingeschlossen und beobachtet. Ziel der Studie ist es die Erkennung und Biologie der Abstoßungsreaktion besser, sowie früher darzustellen.

# Abstract

## Aims

The aim of this pilot-study is to explore the utility of liquid biopsies for monitoring transplant failure. Clinically transplant failure presents as tissue damage of the transplant due to ischemia, reperfusion injury or immunological rejection. Cell death in the transplant is associated with release of graft-derived cell-free DNA and microRNA from damaged tissue. Accordingly, graft-derived cfDNA/cfmicroRNA can possibly be used as a minimally invasive tool for assessment of transplant damage. On the other hand, immunological rejection could be associated with clonal expansion of T-cells targeting the graft.

## Methods

### Patients and sample collections

From August 2018 till August 2020, a prospective study will be performed at the Medical University of Graz. In this study, approximately 200 patients with organ transplantation will be included. Blood samples will be obtained before transplantation, at Day0, Day7-10, Month 28(+/- 7 days), Year1(+/- 1 month) and if the therapy regime changes or a biopsy is needed.

### Preparation of Nucleic Acids & White-blood cells

Cell-free DNA is extracted with the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and allelic profiling is performed using the HID-Ion AmpliSeq Identity Panel. MicroRNA is prepared using the miRNeasy Plasma/Serum Advanced Kit (Qiagen, Hilden, Germany), DNA of mononuclear white blood cells is used for PCR of CDR3 region with specific VDJ-region primers (1).

## Results & Conclusions

This pilot study demonstrated the feasibility of comprehensive analysis from RNA and DNA with pilot probes from the established trial. cfDNA can be discriminated up to 0.1% of donor DNA in the recipient´s blood stream. microRNA-profile as well as decrease in TCRB-variability may hint to transplant rejection. We gained approval

from the ethics commission at the Medical University of Graz to proceed to a full clinical study. Starting in September 2018 all patients receiving kidney or liver transplantation at LKH Graz will be invited to participate. 3BR aims to collect and analyze consecutive blood samples of 160 Patients over an observational period of 2 years after transplantation. We hope to improve patient health by supporting clinical decisions with information on the emergence and nature of organ rejection.

# Table of Contents

# Glossar und Abkürzungen

| | |
|---|---|
| SOT | Solid Organ Transplantation |
| 6-MO | 6-Mercaptopurine |
| DBD | Donation after Brain Death |
| ESRD | End-stage Renal Diseases |
| DDKT | Deceased Donor Kidney Transplants |
| LRDKT | Living Related Donor Kidney Transplants |
| ECMO | Extra Corporal Membrane Oxygenation |
| LT | Liver Transplantation |
| ESLD | End-stage Liver Disease |
| SLT | Split Liver Transplantation |
| LLS | Left Lateral Segment Graft |
| HLA | Human Leukocyte Antigen |
| MHC | Major Histocompatibility Complex |
| MBP | Mega Base Pair |
| CD | Cluster of Differentiation |
| APC | Antigen Presenting Cell |
| LFT | Liver Function Test |
| PT | Prothrombin Time |
| ALT | Alanine Aminotransferase |
| AST | Aspartate Aminotransferase |
| CK | Creatinine Kinase |
| EBV | Epstein-Bar-Virus |
| cfDNA | cell-free DNA |
| rdcfDNA | Recipient-derived cell-free DNA |
| gdcfDNA | Graft-derived cell-free |
| ddcfDNA | Donor-derived cell-free DNA |
| CSF | Cerebrospinal Fluid |
| NGS | Next-Generation Sequencing |
| SNP | Single Nucleotide Polymorphism |
| TCR | T-cell Receptor |
| CDR | Complementary Determining Regions |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| RACE | Rapid Amplification of cDNA Ends |
| ORF | Open Reading Frame |
| dATP | Desoxy-Adenosintriphosphate |
| FACS | Fluorescence-Activated Cell Sorter |
| LOD | Limit of Detection |
| LOB | Limit of Blank |
| LOQ | Limit of Quantification |
| RIN | RNA-Integrity Number |
| RNA | Ribonucleic Acid |
| PCA | Principal Component Analysis |
| FU | Fluorescent Unit |
| ddPCR | Digital Droplet PCR |

# List of figures

# List of tables

# 1 Introduction

## *1.1 Organ Transplantation*

At the end of 2017 over 500 patients wait for a kidney transplant, over 60 are in need of a liver donation – same for heart transplantation – and over 100 patient names are written down on the lung transplant waiting list in Austria (2). There are ten-thousands of people living with a donor organ in Europe and the numbers will be continuously rising in the future. Due to our ageing society and the higher prevalence of chronic diseases we may slide into a shortage of possible donor organs. Therefore, it is a top goal to treat transplanted patients in a risk-reduced manner and to monitor them for a possible transplant rejection. Furthermore, it would be a huge improvement to detect rejection in an early stage so that chances for preserving the organ are much higher (3).

### 1.1.1 History of Organ Transplantation

The era of organ transplantation began in the early 20th century. Surgical skills and techniques had been widely improved, based on animal auto- or allo-transplantations (dog-to-dog or dog-to-goat). This led to the development of solid organ transplantation (SOT) with applications on humans by transplanting animal organs into humans, called xenotransplantation. For this surgical intervention the technique of vascular anastomosis and sutures had firstly be mastered and were carried out first by Jaboulay and Carrel. Alexis Carrel actually was granted with the Nobel Prize in 1912 for pioneering the vascular suturing, which he actually got taught from Mathieu Jaboulay (4). They first successfully transplanted dog-goat xenografts and later performed the first xenotransplant in humans in 1906. Jaboulay treated two patients with renal failure by transplanting a goat kidney into one and a pig kidney into the other; in both cases, he anastomosed brachial and renal vessels. The transplants failed and both patients subsequently died, but there was no alternative treatment option. Without the intervention, death was immanent, because those patients had already been in an terminal stage of renal failure (5).
After showing the possibility for a SOT at this time more and more transplantation procedures emerged – as for example skin grafts which were mainly autografted tissues. These approaches led to the observation that failing of those grafts were

mainly based on lymphocytes. Getting rid of immune cells with irradiation or splenectomy for example decreased the failing of the skin homograft (4).

In deduction of these findings Medwar was the first who described the need of immunosuppressive therapy to maintain the graft function for homografts (6). In 1954 a reported kidney transplantation between identical twins showed a sustained long-term function of the organ and further supported the immune mediated rejection theory.

Back then it was the beginning of the so-called rise of the conventional immunosuppression drugs, mainly based on steroids. First tested on dogs the drug 6-mercaptopurine (6-MO) and its pharmacological derivatives led to longer graft survival as this was shown by Thomas Starzl and his colleagues (7). This led to the improvements that modern organ transplantation protocols basically still utilizing, namely human leukocyte antigen (HLA) genotyping or strictly use of immunosuppression protocols with high-dose regimens if a transplant rejection is suspicious (7).

Even though the therapy was suboptimal - out of a present point of view - for transplanted patients and resulted in high rejection rates and early graft failure, it improved the overall survival significantly back then and it proved the immune mediated rejection theory.

The next step in transplant history marked the advancements in the field of transplanting organs of deceased donors. Out of the SOT group, only kidney transplantation could be performed with living donors, so other organs were dependent on corpses and on a diagnosis where the organ is mainly preserved but the human itself already died. Despite the ethical issues, the so-called concept of “brain death” mainly fits for these points. Criteria like coma, absence of brainstem reflexes and apnea were firstly used for this diagnosis (8). The subsequent donation after brain death (DBD) results in better graft survival due to less perfusion leakage of the transplanted organ. Those patients normally have a prevailed circulation and therefore the organ remains physiologically perfused in comparison to a donation after circulatory death (DCD) where the organ often suffered from a perfusion damage and necrosis, which then leads to worse outcomes (9).

### 1.1.2 Kidney Transplantation

Kidney transplantation is one of the few SOTs which can be carried out between two living humans, called living kidney donation.

Firstly done in 1954 in the Peter Brent Brigham Hospital in Boston the technique is now successfully used in the routine and reflects an indispensable pillar in treating end-stage renal diseases (ESRD) (10). Only the dialysis – blood- or peritoneal dialysis – is a possible alternative treatment for ESRD, but it will result in lower overall survival, if treated for several years. The worth-striving goal is a SOT. As said before there are two different ways, which are reflecting the paths to get a donated kidney. On the one hand there is the deceased donor kidney transplantation (DDKT) which basically means that the patient is registered on a waiting list for an organ which is donated by a deceased human, for example due to brain death. In Europe some countries are enclosed to a non-profit organization called Eurotransplant which is coordinating an international organ transplantation network that facilitates patient-oriented allocation of deceased donor organs. This leads to faster and improved results, caused by higher organ availability and higher HLA-matched organs that can be found for the potential recipient and this further leads to better engraftment and graft survival (11). Nowadays one can go even further by additionally matching weight and sex, which leads to decreased risk in early graft loss (12). The post-mortal organ donation by Eurotransplant is executed by strict and transparent criteria. For example, the time on the waiting list and the HLA-matching playing important roles for the allocation of the organ. Furthermore, the distance, as Eurotransplant is a pan-national organization, is taken into consideration. This results for example in an overall mean waiting time for about 6-10 years for a kidney transplantation (2).

On the other hand, it is possible to get a kidney donated from a living human, also called living related donor kidney transplant (LRDKT). Nearly every third kidney transplantation nowadays is transplanted out of a living donor (13). In reminiscence to the first transplantation ever done, which was also a LRDKT, it is now proven that there are some advantages for this form of transplantation. The first one is that it is possible to test, match and choose the perfect or a nearly perfect donor which reduces downstream the usage of immunosuppressive drugs. Even further the organ has not to be transported from the cadaver to the patient like in DDKT, so the

time of ischemia for the transplanted organ is much lower, which results in a better 5-year prognosis for living donor transplantation (14).

Due to the improved results kidney transplantation is prone to be involved in ethical and moral issues. First there is a unequal access to living donors worldwide and furthermore countries are in service for not creating a black market for organs, especially in kidney transplantation (15). Ethic committees are normally involved in that kind of decisions to rule out the abuse of financial interests. Despite the fact that more living donors are related to each other, there are also altruistic motives to donate a kidney to a nondirected recipient, which involves unknown persons. As said before this honorable behavior might look, in the first place, virtuous but digging deeper it may be questionable what the intents of the donor are, or if there is a normal mental health status as this kind of nondirected donation may have occult reasons (16). A donation is not a no-risk procedure for the donor. In the literature there is kind of a post-operation fatigue-syndrome described, which leads to reduced fitness of patients after donation. Also surgical related side effects must be taken into account like wound healing disorders or even a renal insufficiency or hypertonia (17). The overall risk for death during the organ transplantation in state-of the art hospitals is under 1 ‰ (18).

The operation is basically the same for the recipient independent of LRDKT or DDKT. Basically, it consists in implantation of the donated kidney into the heterotopic *fossa illiaca*, which is in contradiction to other SOT like liver or heart which normally transplants to the orthotope location in the human body. During the operation the ureter is connected to the *vesica urinaria*. The reason why it is done on the heterotopic side is to get easier access to the *arteriae* of the pelvis (19,20).

After transplantation the recipient has to be treated with an immunosuppressive therapy to prevent transplant rejection. Nowadays the protocol normally consists of a double or triple therapy with calcineurin-inhibitors (like Ciclosporin or Tacrolimus), a proliferation inhibitor (like Mycophenolate-mofetil or Azathioprine) and a glucocorticoid (e.g. Prednisolone). The dosage is not fixed, beginning with a higher concentration and leading to a dose reduction after time, to minimize side effects of the immunosuppressive therapy. Patients have a higher risk developing malignant diseases and metabolic diseases. Furthermore, Tacrolimus and Ciclosporin itself are nephrotoxic so there is a fine line of dosage which may be exacerbated by suspected organ rejection. There are also modern immunosuppressive drugs like

IL-2α antibodies and mTOR-inhibitors which circumvent some kind of the classical side effects but may induce others (21).

The before mentioned network involved in coordination of organ transplantation is called Eurotransplant. Eurotransplant consists of Austria, Belgium, Croatia, Germany, Hungary, Netherlands, Slovenia. In 2018 in Austria 134 kidneys have been donated and used for transplant from deceased donors through the Eurotransplant network (Table1). In 2018 the active waiting list in Austria consisted of 597 patients at the end of the year. The total count for patients waiting for kidney organs in the whole Eurotransplant network was 10791 at the end of 2018 (22).

This reflects the need for kidney organ donation and the need to improve the engraftment and graft survival after donation to not lose any valuable and rare organs.

**Table 1: The table shows the actual counting of individual transplanted organs of deceased donors from the Eurotransplant network divided into contributing countries (22).**

| Deceased donor organs used for transplant, Jan-May 2019 / 2018, by donor country | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B | D | H | HR | L | NL | SLO | Non-ET | Total |
| 2019 | kidney | 119 | 178 | 638 | 119 | 61 | 4 | 153 | 23 | | 1295 |
| 2018 | kidney | 134 | 201 | 675 | 147 | 68 | 5 | 214 | 22 | | 1466 |
| 2019 | heart | 29 | 39 | 131 | 30 | 11 | 1 | 17 | 6 | 5 | 269 |
| 2018 | heart | 31 | 29 | 121 | 32 | 15 | 1 | 12 | 6 | 3 | 250 |
| 2019 | lung | 69 | 86 | 249 | 22 | 7 | 2 | 66 | 2 | 28 | 531 |
| 2018 | lung | 70 | 98 | 275 | 37 | 8 | 2 | 57 | | 22 | 569 |
| 2019 | liver | 58 | 109 | 306 | 37 | 44 | 1 | 63 | 8 | 5 | 631 |
| 2018 | liver | 64 | 125 | 326 | 48 | 50 | 6 | 77 | 11 | 4 | 711 |
| 2019 | pancreas | 11 | 5 | 46 | 2 | 1 | 1 | 12 | | | 78 |
| 2018 | pancreas | 5 | 7 | 32 | 3 | 1 | 1 | 13 | | | 62 |
| 2019 | pancreas islets | | 4 | | | | | 4 | | | 8 |
| 2018 | pancreas islets | | 4 | | | | | 6 | | | 10 |

### 1.1.3 Liver Transplantation

Liver transplantation (LT) is in contrast to other SOT in urgent need for a patient with an acute liver failure. For a liver, there is actually no replacement therapy that fits the needs of a fully functional liver. For replacing the function of a kidney, one may use dialysis, for lung and heart it is possible to use an extra corporal membrane oxygenation (ECMO) to provide cardiac and respiratory support. The options for a liver replacement therapy are all experimental and none of them mimics all of the functions, that a liver physiological has to fulfill (23). After Starzl's first approach to transplant a liver in 1963 improvements led from a 1-year survival of under 10%, to a 5-year survival of over 80% (7,24). Indications for a liver transplantation are highly diverse and spanning from inherited malformations (mainly in newborn and children), metabolic diseases to alcohol and infections, that destroys the liver in a chronical manner. Also, malignant processes like the hepatocellular carcinoma are eligible for transplantation, but may be assessed through the Milan Criteria (25). The urgency for the transplantation is assessed through a model for the end-stage liver diseases (ESLD) called the MELD-Score. The acronym MELD means model for end-stage liver disease and it was created to undergo a prediction for survival of the patient, without the in the last term life-saving transplantation, to distribute the liver to those patients with the highest need (26).

All of these above-mentioned criteria showed the need for LT but are also reflecting an imbalance of donors and recipients. This led to the technique of the so-called Split liver transplantation (SLT). This technique is an important strategy to minimize the need for liver donors, by creating two transplants out of one allograft. The liver itself has a high self-renewal capacity and by splitting the bipartite liver into two hemi-grafts, a left and a right segment, two possible patients and diseases could be cured. Normally the smaller left lateral segment graft (LLS) is transplanted to a child and the larger right lobe into an adult (27).

The surgical intervention itself is an orthotope transplantation and consists of a large abdominal incision and vascular anastomosis of vena cava and portal vein in a physiologically right manner (28).

The cold ischemia in liver transplantation is a huge problem and techniques for liver preservation, like a machine perfusion are not far from common usage. The machine perfusion is made to overcome the conventional cold storage when transporting the

organ. This technology provides evidence to reduce organ damage due to longer distances (29).

The waiting list from Eurotransplant reflects the need for liver transplantation. 85 people in Austria are awaiting a liver transplantation at the end of 2018 (Table1). The smaller number in comparison to kidney transplantation reflects the urgency of liver transplants as most of the patients have a low, with nearly going to zero, 1-year survival without a transplant. Overall 1390 patients in the Eurotransplant network are awaiting a liver (30). This reflects the need for patient's surveillance in order to avoid a transplant rejection after LT which often results in worse outcomes as a retransplant may be prolonged due to limited organ donors and ultimately results in patient's death.

## *1.2 Transplant rejection*

When an organ is transplanted the host´s immune system will detect foreign antigens which leads to the destruction of the transplanted tissue. This process is called transplant rejection and reflects one of the major hurdles in transplantation. An immune evasion can be performed by matching the donor and the recipient's HLA-genotype, but a full match is, most of the time, not feasible in the routine.

The first transplant rejection showed Medawar in 1944 in skin allografts (6). Later on, in the era of immunosuppressive drugs in transplantation the overall survival drastically improved and a graft survival was the common, not the exception.

### 1.2.1 Immunology of Transplant Rejection

In a SOT setting, immunological aspects are mostly contributing to transplant rejection. Basically, the major histocompatibility complex (MHC), or as a gene region also called in humans the human leukocyte antigen (HLA) – system is fundamental for the understanding of transplant rejection. The HLA system comprises a set of genes which is involved in detection of foreign antigens. It is part of the acquired immune system and antigens are presented through this system to the T-cells. It consists of a transmembrane domain and a larger extracellular domain. All extracellular domains are glycosylated (31).

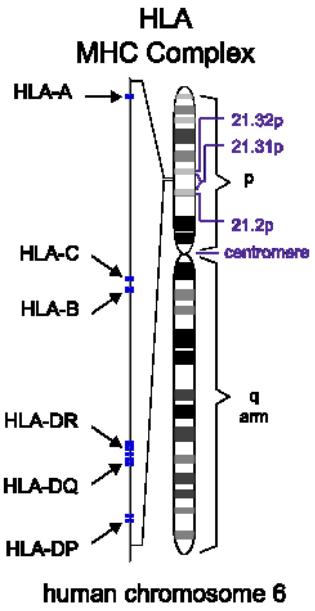


**Figure 1: HLA coding regions. The human chromosome 6 spans more than 170 million base pairs and contains the MHC or HLA loci. The most important loci are marked with arrows (32).**

HLA genes are based on chromosome 6, specifically on the petite arm and spans from 6p22.1 to 6p21.3, with about 29Mb to 33Mb on the hg38 genome as the reference assembly.

The HLA class itself is a highly polymorphic region and there are over 100 common alleles known for HLA-B and over 70 common alleles for HLA-DRB1 (33,34). The whole diversity can be seen in the IMGT-HLA database. There are, for example, over 5000 different alleles for HLA-B (35).

The MHC-System can be segregated into three different classes, which are different in structure, function and expression on different kind of cell types or tissues. MHC class I and II molecules are glycoproteins and class III MHC molecules are mainly regulating proteins part of the complement system.

**Table 2: Diversity of HLA Class I alleles in the IMGT database (36).**

| HLA Class I | | | | | | |
|---|---|---|---|---|---|---|
| **Gene** | **A** | **B** | **C** | **E** | **F** | **G** |
| **Alleles** | 5,018 | 6,096 | 4,852 | 30 | 44 | 68 |
| **Proteins** | 3,398 | 4,248 | 3,193 | 9 | 6 | 19 |
| **Nulls** | 272 | 204 | 222 | 1 | 0 | 3 |

**Table 3: Diversity of HLA Class II alleles in the IMGT database (36).**

| HLA Class II | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **DRA** | **DRB** | **DQA 1** | **DQB 1** | **DPA 1** | **DPA 2** | **DPB 1** | **DPB 2** | **DMA** | **DMB** | **DOA** | **DOB** |
| **Alleles** | 7 | 2,923 | 149 | 1,56 | 106 | 5 | 1,36 | 6 | 7 | 13 | 12 | 13 |
| **Proteins** | 2 | 2,087 | 53 | 1,046 | 38 | 2 | 895 | 3 | 4 | 7 | 3 | 5 |
| **Nulls** | 0 | 105 | 5 | 58 | 1 | 0 | 69 | 0 | 0 | 0 | 1 | 0 |

**Table 4: Diversity of HLA Class II – DRB alleles in the IMGT database (36).**

| HLA Class II - DRB Alleles | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **DRB1** | **DRB2** | **DRB3** | **DRB4** | **DRB5** | **DRB6** | **DRB7** | **DRB8** | **DRB9** |
| **Alleles** | 2,403 | 1 | 217 | 108 | 77 | 3 | 2 | 1 | 6 |
| **Proteins** | 1,759 | 0 | 175 | 83 | 70 | 0 | 0 | 0 | 0 |
| **Nulls** | 105 | 0 | 8 | 13 | 8 | 0 | 0 | 0 | 0 |

Class I MHC molecules are expressed on all nucleated cells, but with different receptor density on the cells surface. Only red blood cells, where enucleation took place in the hematopoiesis, do not express MHC I. MHC 1 molecules consist of two different polypeptide chains, a highly polymorphic alpha chain which consists of (α1, α2, α3) and a constant region which is called the beta-chain and it is part of the ß2-microglobulin which sits on the chromosome 15. The most prominent regions are A, B, C, but other alleles are also possible (E, F, G). MHC binds to endogenous peptides and presents those antigens on the cells surface to the corresponding T-cell receptor of cytotoxic T-cells (CD8+ cells). Peptides binding to the MHC are mainly processed in the corresponding cells proteasome and degraded products of cytosolic proteins. In the endoplasmic reticulum those MHC receptors and those peptides then form a complex, which is then presented at the cells surface. With a specific amino acid chain that spans from position 240 to 245 they interact with the main CD8 Receptor, but other smaller co-binding factors and receptors are involved as well. If the receptor is loaded with a foreign antigen e.g. when the cell is infected by a virus, then the MHC binds to the cytotoxic T-cell and an immune reaction is triggered, which results in death of the infected cell due to the CD8+-T-cell. Cells may evade of these immune attack by downregulating the MHC I but since the receptor is present in all cells, evolution brought up the NK-cell fraction which are scanning for downregulated MHC I, and if there is a disbalance in receptor density,

the NK cell will induce cell death of the target cell due to FASL-FAS binding, which is basically the “death kiss” for the cell and leads to apoptosis (37,38).

Class II MHC molecules are present on the antigen presenting cell (APC) fraction. These cells are also called immunological active cells and include dendritic cells, monocytes/ macrophages, B-lymphocytes, vascular endothelial cells and activated T-cells. These cells are able to express MHC II due to cytokine stimulation. Class II MHC molecules are built up out of two different chains (α and ß) which are not covalently bind to each other. The corresponding coding gene region is polymorph and leads to high diversity (Table 3,4). MHC II are able to bind exogenous proteins or peptides, in order to present them to cells of the adaptive immune system e.g. CD4+-T-cells (T helper cells). APC cells taking up these antigens and present them to T-cells in the lymph node for example. The epitope of the MHC II binds to the corresponding paratope of the T-cell and will then induce an immune reaction. This binding happens in the first place to so-called naive T cells, which then, due to the specific microenvironment that is produced, mature and may also involve a B-cell response (38).

Class III MHC molecules are encoded of three different gene regions (C4, C2 and BF). They are responsible for complement factors and other cytokines (TNF-α and TNF-ß) (39).

The great allelic variability of MHC molecules results in diverse gene sets in humans. This diversity then results in the problems for finding a specific donor or a matching recipient-donor pair which then leads to a higher risk for transplant rejection (40,41).

There is also a so-called minor histocompatibility complex which may further induce a reaction against the graft in a fully matched HLA pair of recipient and donor (42,43).

As being said, the risk for a transplant rejection is mainly based on genetically disparity or less matching between the HLA-type of the recipient and the donor – this also includes the time and speed for this immune attack to happen. The responsible CD8+ cytotoxic T-cells and CD4+ helper T-cells are orchestrating this attack.

The rejection itself then may form or present its pathology in different patterns: hyperacute, acute and chronic transplant rejection (44).

In acute transplant rejection a stimulation of CD4+ helper t-cells of the recipient, induced by the HLA antigens of the transplanted organ, leads to a release of cytokines (IL-2, IFN- γ, TNF- α) and an induction of cell adhesion molecules (45,46). IL-2 for example activates cytotoxic T-cells and attracts them to the organ. Binding of cytotoxic T-cells leads to the release of damaging effector molecules, like the perforin which then destroys the membrane of the target cell and enables proteases (serine-proteases) and granzymes to start apoptosis (45,47–49).

IFN- γ activates monocytes/macrophages and leads to phagocytosis and lysosomal activity, which then enhances a respiratory burst against cells and induces the release of TNF-α. Intercellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) are attracting circulating leucocytes in the endothelium of vessels to start a process called diapedesis (46–48).

A chronic transplant rejection occurs over a time span of months and years. It is the main reason of graft failure over time and limits the lifespan of a graft. There are other reasons which also may lead to graft failure like chronic inflammation (e.g. hepatitis or CMV) or missing compliance of the patience (48). Other factors like sex, age and weight of the donor, as well as the underlying pathology in the recipient are also playing a role in chronic transplant rejection. The immunological interaction between the recipient’s immune system and the donor organ maintains chronic inflammation and rejection. This leads to a vasopathia in the transplant organ’s vessels and may lead to fibrosis and atrophia due to ischemia (48).

A hyperacute rejection happens when preformed antibodies in the recipient are ready to attack the organ, short time after transplantation or more precisely, short time after the donated organ is connected to the recipient’s blood stream and blood, including antibodies, streams into the vessels. Those preformed antibodies have been formed, if the recipient has already contact, in some way, to the foreign HLA type, which resulted in expression of immunoglobulins directed against this HLA-type. This could be due to former transplants or blood transfusions (46,50).

Not all organs are prone to suffer from HLA mismatching. Best evidence supports matched HLA-types for renal and bone marrow transplantation. Matching allotransplants have a higher graft survival and less chance to get rejected.

It is questionable if matching has the same evidence for other organs. like liver, but there may be evidence even for liver too, which can be concluded out of the immunological background of the rejection mechanism (51).

### 1.2.2 Detecting transplant rejection

Traditionally transplant rejection is determined by clinical, biochemical or histopathological (biopsy) evaluation. Current approaches are often lacking in specificity and sensitivity for diagnosing a rejection in an early stage, to prevent further damage of the organ. The histological evaluation under the microscope may be the gold standard, but nevertheless it shows less specificity in early stages. So, in order to reduce the morbidity of graft failure, early detection is key, and surveillance of organ health is essential for a longer graft survival.

In the modern era of diagnostics new biomarkers approached in a variety of scientific fields. Those molecular targets can be found in all “omics” fields and may lead to more robust detection.

The laboratory basic of rejection diagnostic appears to be the conventional parameters for organ damage, in general for the specific organ. For liver this are blood tests that are included in a so-called hepatic panel or liver function test (LFT). LFT includes tests for coagulation (like prothrombin time (PT/INR), aPTT) or parameters synthesized by the livers enzymatical function like albumin or direct bilirubin. The markers for liver damage include aspartate transaminase (AST) and alanine transaminase (ALT). For kidney, chemical parameters like the creatinine kinase (CK) are the most widely used screening parameters for kidneys filtration function and hints to kidney damage (52). Guidelines are representing a comparable way to detect kidney injury. KDIGO (Kidney Disease Improving Global Outcomes) guideline defined a kidney injury as the rise in serum creatinine levels of more than 0.3 mg/dL in 48h, or by a reduction of urine output of lower than 0.5ml/kg/h for more than 6h. Furthermore, one has to test for viral infection like BK/JC polyomaviruses or Epstein-bar virus (EBV). If no culprit can be found a core needle biopsy is indicated and necessary for unexplained levels of serum creatinine. For allograft rejection the Banff-classification provides a standardized criteria in histological diagnosis of acute rejection and scores different parameters like C4d deposition and microcirculation pathologies in renal tissue (44,53).

The immunologic laboratory also plays his role in detection of rejection by determining the count and existence of preformed antibodies that may lead to hyperacute rejection.

All in all, those classical or conventional biomarkers, especially the non-invasive ones are lacking in diagnostic reliability. The serum creatinine for example is a

biomarker that may not change, despite the kidney has already suffered from significant damage. Renal biopsy, even diagnosed within the Banff parameters, can include sampling errors or interobserver variabilities between the diagnosing pathologists (54).

Recently investigated biomarkers are deducted out of findings in the immunology and pathophysiology of transplant rejection. The immune cells secrete chemokines like CXCL9 and CXCL10 that simulate effector pathways in other immune cells, like cytotoxic T-cells which leads to IFN-γ (55). Those molecules show promising results in the use for immune response but also lack in sensitivity for the transplant rejection as immune systems reaction also starts in viral defense (56–58).

Liquid biopsy e.g. has emerged as a potent non-invasive diagnostic tool in cancer and has recently also been applied to transplant monitoring (59). In transplant patient's DNA originating from the transplant or the recipient can be readily discriminated by allelic profiling. Monitoring this circulating donor DNA will provide evidence of tissue damage (60).

In addition to DNA, liquid biopsies have also been shown to contain circulating miRNA (61). miRNA profiling has been used to determine the origin of tissue damage (62,63) and might provide additional information on the mechanism of organ damage in a transplant setting. Monitoring circulating miRNA will provide insights into the location and mode of tissue damage and organ function.

Cell death is a hallmark feature of transplant rejection. Cell death can be the result of reperfusion damage or immune response to the transplanted organ. Houda et al. have demonstrated that the analysis of the T-cell receptor repertoire by next generation sequencing (NGS) provides information about kinetics of T-cell meditated rejection (64). Acute rejection can be inferred from changes in the T-cell clone kinetics with the emergence of transplant specific subclones. Monitoring the T-cell repertoire will also provide evidence of an immunological cause of tissue damage.

#### 1.2.2.1 Cell-free DNA in transplant rejection

The main part of eukaryotic DNA is located within the cell's nucleus. Due to damage of the cell, a part of this DNA disintegrates into the blood stream and reflects the so-called cell-free or circulating DNA (cfDNA). This cfDNA can basically be found in all

kind of body fluids like plasma, urine or cerebrospinal fluid (CSF). Physiologically in the blood stream, the main part of cfDNA is basically from leukocytes as these cells are the most prominent fraction of nucleated cells in the blood. Enzymes present in the blood can degrade those streaming DNA fragments and therefore the DNA itself is limited by a half-life time. The precise half-life time is still unclear, but may be somewhere between minutes and 2 hours (65,66). Clinical applications of cell-free DNA is mainly for the use in liquid biopsy. Monitoring a patient's disease or profile a patient's tumor within the circulating DNA. In this case the circulating tumor DNA (ctDNA) of a single blood sample has some advantages over classical biopsy (67). The non-invasive character of a blood drawing decreases risk and side effects and may allow to monitor the patient over a larger timespan. This is possible because ctDNA is different from normal DNA due to specific mutations of the primary tumor. This allows diagnosis of molecular mutations and even tracking of tumor burden. Tumor burden basically means the amount of ctDNA in the blood which may correlates with the therapy effectiveness. An effective therapy against a specific tumor appears as a large decrease of tumor mass – therefore it results in a high amount of ctDNA – and after that, in only low to none ctDNA. By tracking the amount, it is possible to see relapse or therapy failure earlier in comparison to other methods (68). In transplant patient´s there is a kind of unique situation because cfDNA of two different genetic backgrounds is released into the blood stream. There is the presence of the recipient-derived cell-free DNA (rdcfDNA) and the graft-derived cell-free DNA (gdcfDNA), or in the literature also often called donor-derived cell-free DNA (ddcfDNA). This phenomenon was first reported in 1998 by Lo et al. (69). After the coming of age of next generation sequencing the cell-free DNA developed to a usable biomarker for transplant rejection (70,71). Past research attempted to detect a rejection within the borders of the Y chromosome. A female recipient with a donated male kidney has gdcfDNA, that consists a Y chromosome which functions as a donor genetic signature. This is possible in the blood or ,for kidney patients, in the urine (72). This special case when women received a male organ is obviously limited and research moved on to other individual biomarkers that allowed to distinguish between recipient and donor. A sex-independent marker is a single nucleotide polymorphism, abbreviated called SNP. It is possible to distinguish any human individual, regardless of sex, by this unique pattern of polymorphisms (73). A SNP is defined as a substitution of a single nucleotide at a specific genomic

position. The frequency of a SNP in a population is >1% in allele frequency. Single nucleotide polymorphisms have been used successfully for tracking heart and lung transplant rejection (70,74). A list of studies which have been performed using cfDNA as a test for transplant rejection, is listed in Table 5. Recently a systematic review demonstrated that the ddcfDNA, which typically varies between 0,3% and 1,2% of the whole amount of cfDNA in the blood stream, can discriminate between active rejection or stable organ function in kidney transplant recipient (75,76). The cfDNA, as all new biomarkers, has to compete with the gold standard, which is represented by biopsies. A biopsy with the indication for detecting transplant rejection has to be carefully weighed up against the side effects. A kidney biopsy has a major complication chance for gross hematuria. In a large series of biopsies patients developed hematuria with a rate of 1% (77). A new biomarker in this field has to be safe and acceptable to use and detect rejection with a sensitivity or specificity as high or nearly as high as the gold standard, or to even outperform this. What also has to be taken into consideration is that a biopsy in as high as 25%, leads to a non-reliable detection due to inadequate specimens. This number may increase with smaller needles. The change in immunosuppression therapy regimes may also lead to infrequent detection of a sub-clinical rejection (78). Due to these reasons new biomarkers are in need to compensate the disadvantages in the histological testing.

When testing cfDNA it is important to know that cfDNA levels appear to vary by organ. The highest mean fraction is measured in the liver (mean cfDNA of 3.3-5%) followed by lung (2-5%) and kidney (0.3-1.2%) (79). The stability of cfDNA over a time course firstly peaked after transplantation and subsequently falls into steady state level mentioned before around 7-10 days posttransplant.

**Table 5: This table shows a review of clinical studies performed using the cfDNA as a test for transplant rejection. The studies are categorized into specimen type, biomarker and methodology used for the detection.**

| Post-Transplantation DNA biomarkers of transplant rejection | | | | |
|---|---|---|---|---|
| **Organ** | **Sample** | **Biomarker** | **Methodology** | **Reference** |
| Kidney | Urine | ddcfDNA (SNP) | qPCR | Sigdel et al. (80) |
| Heart,Lung | Blood | ddcfDNA (SNP) | qPCR | De Vlamnick et al. (70,74) |
| Heart | Blood | ddcf Y-chromsomal DNA | ddPCR | Snyder et al. (71) |
| Liver,Kidney,Heart | Blood | ddcfDNA (SNP) | ddPCR | Beck et al. (79) |
| Kidney | Blood | ddcfDNA (SNP) | mmPCR | Sigdel et al. (80) |
| Kidney | Blood | ddcfDNA (SNP) | ddPCR | Bloom et al. (76) |
| Liver | Blood | ddcfDNA (SNP) | ddPCR | Schütz et al. (60) |
| Heart | Blood | ddcfDNA (SNP) | ddPCR | Khush et al. (81) |
| Kidney | Blood | ddcfDNA (SNP) | ddPCR | Huang et al. (82) |
| Kidney | Blood | ddcfDNA (SNP) | ddPCR | Gielis et al. (83) |
| Liver | Blood | ddcf Y-chromosomal DNA | qPCR | Macher et al. (84) |
| Heart | Blood | ddcfDNA (SNP) | ddPCR | Agbor-Enoh et al. (85) |
| Heart | Blood | ddcfDNA (SNP) | NGS | Hidestrand et al. (86) |

#### *1.2.2.1.1 Cell-free DNA in kidney transplantation*

In kidney transplantation, patients' cfDNA can not only be measured within the blood stream. Urine sampling is possible due to direct contact of the transplanted kidney cells to the urine fluid. There is even the possibility to only collect the transplanted organ's urine with surgical implementation of a ureteric double J stent or double-J-catheter (87). Kidney transplant studies, as shown in Table 5, showed that all of it used ddcfDNA to detect rejection either in blood or urine or even both.

#### *1.2.2.1.2 Cell-free DNA in liver transplantation*

In liver transplantation the most important study to detect ddcfDNA in transplant rejection is published by Schuetz et al. Here ddcfDNA was compared with the non-invasive traditional standard - LFT. The results showed a higher sensitivity and specificity in a receiver operator characteristics (ROC) analysis for the ddcfDNA (97.1%, 95% CI 93.4%–100%) in comparision to conventional LFTs (AST: 95.7%; ALT: 95.2%; γ-GT: 94.5%; bilirubin: 82.6%) (60).

### 1.2.2.2 MicroRNA in transplant rejection

#### *1.2.2.2.1 Biogenesis of microRNA*

miRNAs are small non-coding RNAs with a median length of 21-23 nucleotides. They are highly conserved and are involved in gene-silencing and regulation in a post-transcriptional manner.

MicroRNAs were first discovered in 1993 in a model organism for nematodes, Caenorhabditis elegans. The first miRNA that has been identified was "lin-4 RNA" (88) . "lin-4 RNA" seems to negatively regulate the LIN14-protein, which is involved in the transition from the first to the second larval stage of C. elegans (89). The laboratory concluded that lin-4 regulates the lin-14 messenger RNA via antisense RNA-RNA interaction, so that less proteins are expressed (90). With this the starting gun has been fired for the search of other miRNAs. 7 years later Gary Ruvkuns lab found the second miRNA ,"let-7", that is involved in the timing of C. elegans development from the fourth larval stage to the adult (91). And not only in

Nematodes, for example the 21-nucleotide let-7 RNA is highly conserved and can be found in vertebrates and other animals, like the zebrafish, where 48 hours after fertilization the let-7 RNA expression can be detected (92). This was the bone of contention for the investigation of even more microRNAs and a deeper understanding of their function.

The biogenesis of microRNAs involves key proteins from the Argonaut family, abbreviated called AGO-proteins, and two endonucleases called Drosha & Dicer. At first the microRNA is transcribed as part of larger transcript by the POL-II polymerase – The same polymerase which transcribes mRNA. MicroRNAs are then forming a hairpin in this transcript, which is called pri-miRNA. The pri-miRNA is then cleaved by DROSHA, which is part of the microprocessor complex in the cell's nucleus and together with the RNA binding protein DGCR8, which recognizes the hairpin part, it cleaves 1 helical turn downstream from the base of the hairpin. After that, the pre-miRNA hairpin structure is released and through exportin 5 complex it leaves the nucleus. In the Cytoplasm the pre-miRNA encounters DICER, which lops off the loop and this results in a microRNA duplex strand. One of the strands of the duplex is then loaded on the AGO-proteins and together they make the microRNA-induced silencing complex which targets the complementary messengerRNA of these microRNA (93). This AGO-complex binds, most of the time, near the 3´UTR-region and recruits a deadenylase-complex which shorts the poly-A tail and once it is decapped, it results in an degradation of the mRNA (94).

This emerging field of small ribonucleic acids partially extends the transcriptomics and, to keep with the nomenclature mantra, the biological dataset of all microRNAs is called miRNome. Now in 2019, over 400 thousand validated microRNA target interactions (in all organisms) have been experimentally validated (95). And by using the article search of PubMed, with the Search term "("microRNA"[All Fields] OR "miRNA"[All Fields] OR "miRNome"[All Fields])", it has now reached a plateau of nearly 10000 articles in 2017 referring to miRNA.

#### *1.2.2.2.2 MicroRNA in transplant rejection*

In transplant rejection microRNAs may play a crucial role in cellular transcription regulation. In particular Farid et al. have found miR-122 and miR-148a upregulated during an acute transplant rejection in liver transplant patients (96). A vast amount

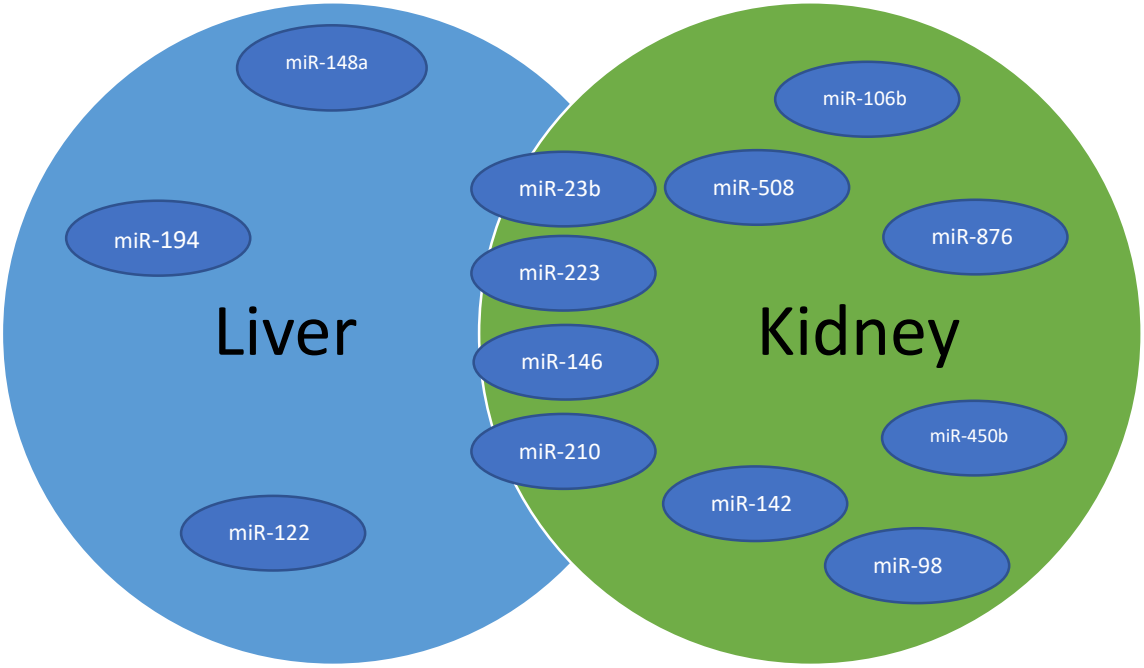


**Figure 2: This Venn-diagram shows the most prominent organ specific and overlapping microRNAs which are believed to be involved in transplant rejection.**

of differentially regulated miRNAs have been detected during the last years, with some dysregulated in specific organ transplantations, while others might even act as pan-organ markers as they were up- or downregulated across two or more organs in a transplant setting. This could be explained because microRNAs are part of some signaling pathways and by changing the transcription of specific mRNAs. During an acute or chronic rejection, the inflammation process orchestrates the organ damage. Different interleukins, mainly IL-6, and TGF-ß are the main mediators and may lead to organ fibrosis, that is clinically highlighted in organ dysfunction (97,98). In fact, microRNAs like miR-548d, miRNA-203 and miRNA-146a are all described as regulators of the SMAD4 protein. SMAD4 itself is a protein that forms homo- and heteromeric complexes and acts a transcription regulator. In

the nucleus it initiates a TGF-ß transcription. If these miRNAs are more transcribed then SMAD4 messenger-RNA will be degraded but these miRNAs all have been described as downregulated in transplant rejection (99–101). Other miRNAs include regulation of T- and B-cell signaling which mainly results in regulation of immune response, lymphocytic infiltration and promotion of inflammation (102,103).

MicroRNAs are mostly encapsulated in exosomes. Exosomes are physiologically occurring, small membrane enclosed micro-vesicles, derived from the endosomal multi-vesicular bodies (MVB) pathway, through reverse inward budding (104). With a diameter of 30-150 nm they are secreted when these MVB fuse with the plasma membrane, a process called exocytosis (105). In fact, early endosomes are developing the so-called late endosomes or multi-vesicular bodies (MVBs), which are characterized by the presence of multiple small phospholipid vesicles inside of the lumen. After the fusion with the plasma membrane of the cell, these small vesicles are released and called exosomes. In these exosomes is then protected against RNA-exonucleases in the blood stream and can be measured through RNA sequencing.

#### *1.2.2.2.3 MicroRNA in liver transplantation*

Liver-specific microRNAs have been discovered in mice and in humans. miRNA-122 for example is a highly conserved liver-specific miRNA. In liver transplant patients' other miRNAs have emerged like miR-122. miR-194 and miR148a are also associated with hepatic damage or declared as hepatocyte-derived. Liver Injury like in liver transplantation showed elevation or downregulation of these miRNAs (96). In Table 6 a more comprehensive list shows the whole spectrum of dysregulated miRNAs in a transplant setting.

**Table 6: This table shows human miRNA expression in liver transplant patients. The asterisk marks no answers given in the publication (61).**

| microRNA dysregulated in liver transplantation | | | | |
|---|---|---|---|---|
| **microRNA** | **Target** | **Specimen** | **Methodology** | **Reference** |
| miR-122, miR-148a, miR-194 | * | Biopsy,Serum | TaqMan miRNA assay | Van Der Meer et al. (96) |
| miR-146a, miR-19a, miR-20a, miR-200a, miR-141, miR-203, miR-20b, miR-205, miR-33a, let-7e, miR-150, miR-34c-5p, miR-342-5p, miR-181c-5p, miR-29a, miR-19a, miR-204, miR-20a, miR-328, miR-1336, miR-223, miR-210, miR-503 | TGF$\beta$R2, SMAD4, EGFR, VGFA, IL-8, IL-6, CCL8, CD40L, IRS2, TLR4, c-Myb, STAT-1, IGF1, VGFA | Biopsy | Affymetrix Gene Chip 2.0 | Brereton et al. (101) |
| let-7c, miR-23b, miR-122, miR-150 | * | Serum | TaqMan miRNA Assay | K. Zen et al. (106) |

#### *1.2.2.2.4 MicroRNA in kidney transplantation*

In kidney transplant patients, it is possible to measure kidney related biomarkers, in this case microRNA through the blood stream or in body fluids like urine. This is the reason that most of the studies, published so far, are done in kidney transplant patients. There are kidney-specific microRNAs like miRNA-146a which also may seem to be a risk factor for transplant rejection. This microRNA is known to be highly elevated in immune response and is involved in signaling of inflammatory pathways. This microRNA has also been mentioned in a recent study, where CD8+-T cells, when treated with IL-15 or IL-2, resulted in a highly upregulated miRNA-146a (107). A more comprehensive list is shown in Table 7.

**Table 7: This table shows human miRNA expression in kidney transplant patients. The asterisk marks: No answers given in the publication (61).**

| microRNA dysregulated in kidney transplantation | | | | |
|---|---|---|---|---|
| **microRNA** | **Target** | **Specimen** | **Methodology** | **Reference** |
| miR-99a, miR-100, miR-151a, let-7a, let-7c, let-7f | * | Serum | miRNA | (108) |
| miR-324-3p, miR-611, miR-654, miR-330 MM1, miR-524∗, miR-17-3p MM1, miR-483, miR-663, miR-516-5p, miR-326, miR-197 MM2, miR-346, miR-658, miR-125a MM1, miR-320, miR-381, miR-628, miR-602, miR-629, miR-125a | RIMBP2, GTDC1, NTRK2, CCDC21, SAMD4B, SYS1, SYNGR1, PTPN9, ETF1, BCL6, ACBR2B, NFIB, RAB11FIP2, ARID4B, PTCH1, HD, HABP4, FOXG1B, H1F0, EDEM1, ZNF673 | Biopsy | Exiqon miRNA microarray | (109) |
| miR-182-5p, miR-21-3p | FOXO1, BCL2 | Biopsy | GeneChip miRNA 3.0 | (110) |
| miR-450b-5p, **miR-142-3p**, miR-876-3p, miR-106b, miR-508-3p, miR-148b, miR-324-5p, miR-98 | TGF$\beta$-1 | B-Cells | TLDA microRNA cards pool A | (111) |
| miR-10a, miR-10b, miR-210 | * | Urine | TaqMan qPCR | (112) |
| miR-25, miR-181a, miR-204, miR-192, miR-10b, miR-142-3p, miR-215, miR-342-3p, miR-615-3p | PRMT5, TP53, CDX2, ATM, HIPK2, TGF$\beta$R1, TGF$\beta$R2, SNAI1, SPDEF, MAD2L1, HRH1, LMNB2, DTL, NCOR2, RAC1, ACVR2B | Biopsy | Whole genome microarrays | (113) |

### 1.2.2.3 T-cell receptor in transplant rejection

The T-cell receptor (TCR) is a protein complex that is anchored on the cells surface and is responsible in detecting antigens that are represented through MHC-molecules. After activation of the TCR, the cells decide to differentiate into a T-helper cell or a cytotoxic T-cell.

The TCR itself has structural similarity to the Fab-fragment of antibodies. The TCR-complex consists of two different protein subunits - over 90% α/β and in approximately 5% γ/δ. These subunits consist of a constant (C) domain and a variable (V) domain, a transmembrane domain and a short c-terminal region. The n-terminal end of the chain, which is part of the C domain spans through the cell's membrane into the cytoplasm and anchors the receptor. Both subunits are extracellular and connected over a disulfide-bridge. The variable domains of the α-subunit consist of V- and J- segments, the β subunit consists out of V- D- and J-regions which are forming the three hypervariable and binding determining regions, called complementary determining regions (CDR). The CDR 2 region interacts primarily with the α-helices of the MHC I and MHC II complexes. The CDR 1 region of the α-chain with the N-terminal end and the CDR 1 of the β chain with the C-terminal end. The CDR3 region contributes the main part in antigen binding. Due to its variability, it determines the binding to the antigen (114,115).

The formation of the TCR is similar to the formation of a B-cell. Both subunits (α/β or γ/δ) are created by the so-called V(D)J-recombination. Precisely the α and γ chains are created through a VJ, the β and δ through a VDJ recombination (V – variable; D – diversifying; J – joining). During the T-cells development the variable region joins with a J and optional with a D region. This is coordinated by recombination activating enzymes called RAG1 and RAG2. This arbitrary recombination leads to enormous diversity to detect a broad spectrum of possible antigens, assumed to be as high as $10^{12}$ (116,117).

The recombination or basically the rearrangement of the genes results in a specific structure for each cell of the variable CDR3 region. Additional nucleotides, which will act as inserts or deletions in this process, are enhancing the diversity even further. The most hypervariable CDR3 region of the TCR is therefore nearly unique, due to the vast amount of possible recombination options and the hypermutation (Indels). By determining this CDR3 region, one can determine the clone itself, so the CDR3 region represents the clone's ability and function. It is possible to

characterize the diversity of the immune repertoire, basically of the αβ TCR cells through highly specific methods on RNA, cDNA or DNA basis (118). The three principle methodologies are the multiplex polymerase chain reaction (PCR), target enrichment or a 5´RACE, which after library preparation will be sequenced by NGS. The multiplex PCR approach is suitable for DNA and RNA. Primers for all different V and J regions are mixed in a multiple PCR approach. The primers are linked to adaptors for sequencing or the adaptors are ligated after the PCR. The target enrichment method also works with RNA, DNA and even with fragmented DNA. Through an end-repair, A-tailing, and ligation of adaptors the sequences are enriched through the use of TCR specific RNA baits. After hybridization of those baits, the target DNA is purified through magnetic beads and forwarded as a library to the sequencer. The third most prominent method is the 5`RACE. RACE means rapid amplification of cDNA ends. The method starts with a cDNA-synthesis through a primer in antisense direction of the coding open reading frame (ORF) of the messenger RNA. The amplified cDNA consists partly of the coding sequence and the full 5´untranslated region (UTR). A terminal nucleotidyltransferase is used to attach desoxy-adenosintriphosphat dATP to the 3´end to introduce a new poly(A) tail. Afterwards an enzyme, called RNase H, degrades the remaining mRNA. The remaining product serves as a template for a nested PCR. With specific primers a new anchor-sequence is introduced to incorporate an adaptor molecule. This product is then used for subsequent NGS.

It is questionable and under debate which method is the best. With a PCR the risk of introducing a bias due to differential amplification exists. DNA, in comparison to RNA, may lead to more robust results, as the RNA sequencing changes with mRNA expression in the cells. But RNA itself shows a deeper understanding in function, because the DNA fragment may not be transcribed (118,119). By using unique molecular identifiers (UMI) it may be feasible to prevent the over- or underestimation of TCR clones due to variable amount of RNA (120). Using a bioinformatic tool or a mathematical model for reducing the amplification bias might also lead to more specific results (121).

Newer methods may enhance specificity by combining fluorescence activated flow cytometry (FACS) with PCR and sequencing (118,122).

### 1.2.3 Therapeutic intervention

The diagnosis of acute transplant rejection is based on clinical and biochemical markers or biopsy. The therapy of acute rejection itself consists of application of high dose methylprednisolone on the three following days. At the same time the standard immunosuppressive regime changes or the drugs will be given at a higher dose. If the rejection is cortisone resistant a anti T-cell antibody therapy over 3 to a maximum of 10 days is indicated. This is one possible regime. Beneath the acute, there is the sneaking chronic rejection which damages the transplant tissue over time due to chronic inflammation. Drug therapy for this type of rejection consists basically of calcineurin inhibiting drugs, like Tacrolimus, to prevent the organ rejection (21,123,124).

Taken together this comprehensive, non-invasive analysis of liquid biopsies in patients with transplant complications may improve patient care and allow an in-time response to organ rejection.

The aim of this work is to explore the possibilities of liquid biopsy for monitoring transplant failure. Clinically transplant failure presents as tissue damage of the transplant due to ischemia, reperfusion injury or immunological rejection. Cell death in the transplant is associated with release of ddcfDNA and microRNA from damaged tissue. Accordingly, ddcfDNA/ cell-free miRNA can be used for assessment of cellular transplant damage, while immune cell repertoire analysis will highlight the cellular immune response directed against the transplant. Furthermore, it could be possible to analyze the function of organs with the microRNA profile in the blood of the transplanted organ.

Furthermore, question raised if early detection through any of these biomarkers indicates an exacerbation of the therapy, to suppress rejection and may lead to a longer overall graft survival.

# 2 Methods

## *2.1 Patients cohort*

For this pilot-study we will use patient blood samples collected for a following full study. In Graz on average 40 kidney transplantations and 60 liver transplantations are performed per year. Approximately 12% of kidney transplant patients and 9% of liver transplant patients experience transplant rejection. We plan to offer study participation to all patients undergoing kidney or liver transplantation during the project duration (n~200) to ultimately reach a cohort size of ~ 20-40 patients with organ rejection which can be contrasted with a similar sized group of patients without transplant rejection.

### 2.1.1 Inclusion Criteria

- Age: Minimum age: 18
- Maximum age: 99
- Sex: Female as well as male participants.
- Patients receiving kidney or liver transplant

### 2.1.2 Exclusion Criteria

- Age under 18 or over 99 years old.
- Pregnant women
- Previous solid organ transplantation (second transplant) or combined organ transplantation
- Immunosuppressive therapy within the past 3 months

### 2.1.3 Sampling

- Blood sampling: a maximum of 240 ml, 40ml per routinely visit and 40ml at additional timepoints are planned

## 2.2 Patients sample collections

From September 2018 till September 2020, a prospective study is performed at the Medical University of Graz, Austria. In this study, approximately 200 patients with organ transplantation will be included. For all patients, clinical data including age, treatment regimen, therapy response, as well as histopathological findings will be retrieved from clinical records. Blood samples will be obtained before transplantation, at Day0, Day7-10, Month 1 (+/- 7 days), Year1(+/- 1 month) and if the therapy regime changes or a biopsy is needed. At each time point 40ml of blood will be collected into cell-free DNA tubes (Streck®, USA) and cell-free RNA tubes (Streck®, USA) yielding a total of 160ml over the full observation period and further 80ml if the additional point is hit. The therapeutic response will be assessed by the clinicians and will not be influenced by our study. To determine the SNP profile of the donor organ tissue samples from the donor (tissue or blood or cells) will be provided by the Department of Blood Group Serology and Transfusion medicine.

## 2.3 Data documentation

Study data as well as patient data will be stored in folders, which are stored in locked locker. For digital data, they are stored on password secured computer, which only authorized staff have access. Each file is separately password secured on the computer to guarantee data security.

## 2.4 Privacy protection

All analyses will be done in accordance to the Austrian Data Protection Act (Datenschutzgesetz). A continuing number will be assigned to all patients (pseudonymized) and stored on a computer with restricted access only for the principal investigators. Data will be processed only with the assigned patient numbers and transferred encoded to cooperation partners. Primary data will stay strictly in our group and will not be forwarded to anyone. When samples will be provided to cooperation partners, they get anonymized samples with information of diagnose, sex and age. Only authorized persons have access to the original data.

## 2.5 Ethics and safety

The aims of this study cannot be achieved with other methods. Participation in this study brings no harm or benefit for the patient. All ethical and safety regulations from the Medical University of Graz as well as the guidelines of the Helsinki declaration will be applied for this study.

This is not a clinical trial, there will be no harm to participants of this study.

All participants of this study, who´s blood samples are collected sign a study specific informed consent. (Anhang – Informed consent). We gained approval from the ethics commission at the Medical University of Graz to proceed to a full study (Anhang – Ethikkomission).

## 2.6 Preparation of Nucleic Acid

The cell-free DNA is extracted with the QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). MicroRNA is prepared using the miRNeasy® Plasma/Serum Advanced Kit (Qiagen, Hilden, Germany) according to the manufacturer. For a normalized quantification a synthetic miRNA Spike-in control of *Caenorhabditis elegans* miR-39 is added prior to the isolation with a copy number of 1x $10^8$. Mononuclear white blood cells will be enriched from whole blood using Ficoll centrifugation and DNA is extracted from Maxwell RSC Blood kit (Promega).

RNA quality check is performed by the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) using the high sensitivity RNA chip. DNA quantification is done with the NanoDrop® 2000 Spectrophotometer (ThermoFisher) or with the Quantus Fluorometer (Promega) using the Quant-iT® PicoGreen dsDNA Assay kit (ThermoFisher Scientific).

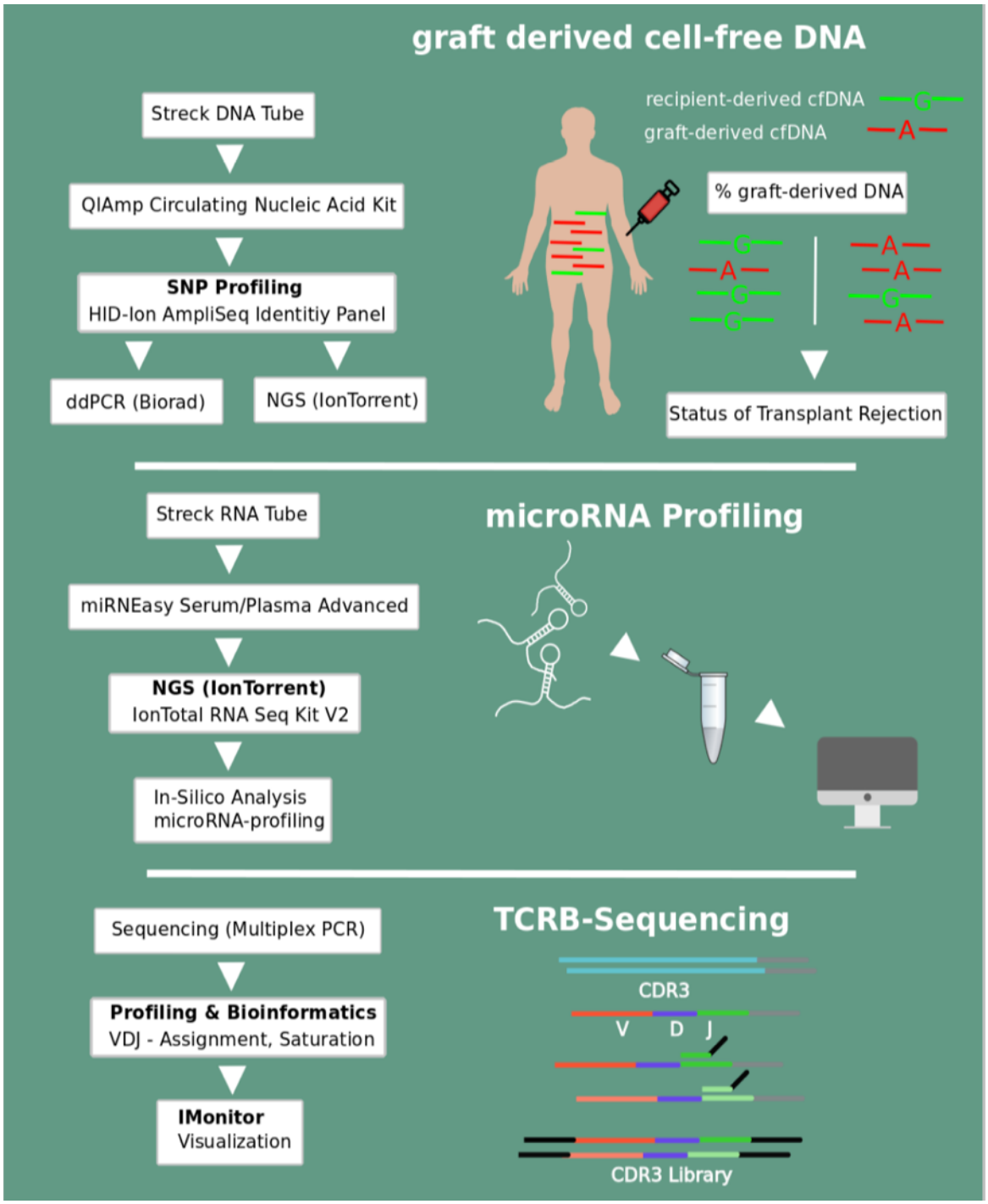


**Figure 3: 3BR aims to detect transplant rejection through a three-tiered approach. From top to bottom: graft derived cell free DNA is isolated and analyzed through a SNP profiling; microRNA is isolated out of plasma and sequenced with the IonTotal RNA Seq Kit V2; For the TCRB sequencing nuclear DNA is isolated and downstream amplified with a multiplex PCR and bias-reducing statistics to eliminate or to decrease confounding effects of the amplification bias. This study aims to compare and to evaluate the three different approaches.**

## *2.7 Sequencing*

Allelic profiling of transplant derived cfDNA will be performed using the HID-Ion AmpliSeq Identity Panel® and through Ion Torrent NGS sequencing. Abundance of donor and recipient DNA will be calculated from allele frequencies of 90 autosomal SNPs and 34 upper Y-clade SNPs. miRNA profiling will be performed by small RNA sequencing using the Ion Torrent NGS Platform using the IonTotal RNA V2 kit. Briefly, adapter molecules are ligated to all RNA molecules and small molecules are subsequently enriched using size selection by Ampure beads. After sequencing data analysis is done using the CAP-miRNA pipeline producing a statistically validated profile of expressed miRNA species. Highly expressed miRNA species will be matched to public databases to determine site and mode of tissue damage. Furthermore 3 normal kidney and 3 normal liver tissue samples, which are provided by the Biobank Graz, will serve as a tissue specific microRNA reference-profile. The tissue samples are prepared through a MagNA Lyser Instrument (Roche). Tissue is lysed for 30 seconds with QIAZOL® lysis agent and homogenized at 6500rpm with beads and immediately cooled on ice. Before sequencing, miRNA amount was analyzed with the Bioanalyzer (Agilent) and the appropriate High Sensitivity RNA chip.

Mononuclear white blood cells will be enriched from whole blood using Ficoll centrifugation DNA will be extracted using Roche Magnapure or RSC Blood DNA kit. The T-cell repertoire will be analyzed by universal multiplex PCR amplification of the variable region of the T-cell receptor followed by NGS sequencing with (1). Library preparation was either done with the AmpliSeq LibraryKit 2.0 (Qiagen) or with the Ion Plus Fragment Library Kit (Thermo Fisher Scientific). T-cell clone kinetics will be compared to cfDNA abundance and miRNA profile, to determine T-cell mediated transplant rejection.

After DNA Isolation a multiplex PCR is used to amplify the V(D)J region. Primers in Table 11 are used for the multiplex PCR. Oligonucleotides (Primers) were ordered from Eurofins (Germany). Multiplex PCR conducted with the Ion AmpliSeq LibraryKit 2.0 (Qiagen) has the following PCR parameters: 21 cycles (2min 99°, 15sec 99°, 60sec 65°, hold 10°). After PCR the preparation is proceeded according to the manufactures protocol.

Sequencing is performed on the Ion Torrent semiconductor platform. MicroRNA and SNP analysis was performed with the 200bp workflow using IonProton P1 or IonS5 chips. The TCRB sequencing was performed using the 400bp workflow on the S5 530.

## *2.8 Bioinformatics*

Cell-free DNA analysis of the SNP profile is done through our in-house pipeline consisting of SnpEff, which annotates the SNP variants. Further processing of the data is done with R.

MicroRNA analysis is done within the CAP-miRSEQ pipeline of the Mayo Clinic. We used the miRbase Version 28 and aligned with the hg38 (Figure 4, 125). Basically, in the CAP-miRSeq pipeline raw reads are trimmed and aligned to a reference genome. Those aligned sequences then may also be aligned to the miRbase with the

miRDeep2 module. Known as well as novel miRNAs can be detected. TCRB analysis is done with the iMonitor pipeline (1). This tool provides a re-alignment and

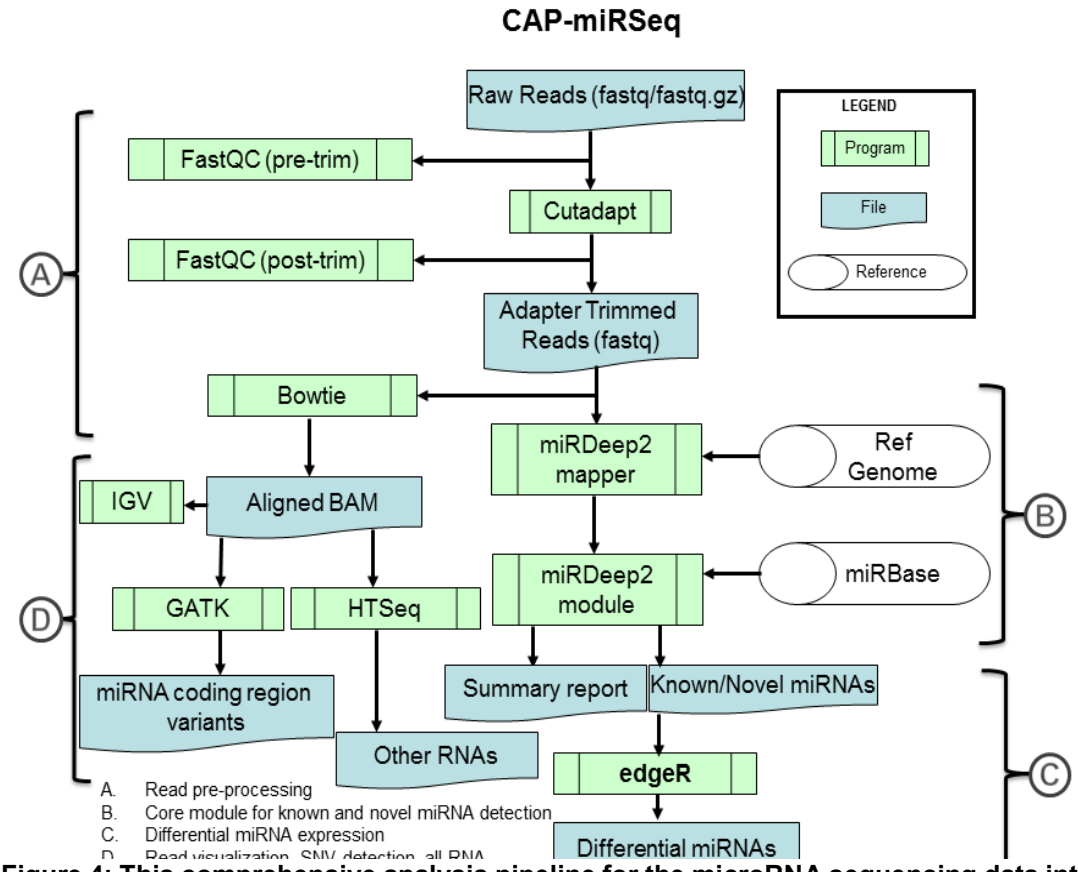


**Figure 4: This comprehensive analysis pipeline for the microRNA sequencing data integrates a pre-processing, alignment and a novel miRNA detection and quantification. All data is partly visualized with R (taken from 125).**

an identification of V(D)J alleles after a common local alignment. Also, a possible bias introduced by the multiplex PCR is minimized and corrected.

## *2.9 Statistics*

Based on the biomarker profiles obtained from controls, organ recipients with and without rejection at different time points analysis will focus on the ability to detect and quantify organ failure, tissue damage and organ rejection by means of miRNA content, abundance of donor DNA and white blood cell profile. Support is provided the Institute for Medical Informatics, Statistics and Documentation of the Medical University of Graz. Graphics. Tables and statistical tests are produced with R packages in RStudio or with Microsoft Office Excel 365.

# 3 Results

After gaining ethical approval probes were collected and a first pilot study was conducted, following the protocol described in the methods part. The first collected specimens served as a first batch to evaluate and to prove the methodic part. The goal is to prepare and establish methods for the full evaluation after the end of the clinical part.

## *3.1 Cell-free DNA – SNP profile*

To establish a cell-free DNA analysis out of blood we choose to use cell-free DNA BCT® (126) tubes as these tubes are stable up to 14 days at room temperature. This allows an easy use for the clinicians to draw blood and further to send the blood tube to our laboratory, without cooling or timing problems that may affect our results.

The first part was to explore a limit of quantitation for our cell-free DNA SNP profiling. In order to mimic the in vivo mixture of donor and recipient DNA we mixed plasma of known concentrations and from two different patients in ratios of 1:10, 1:100, 1:1000 and 1: 10000 – that will mimic 10% donor DNA in the blood stream, 1%, 0,1% and 0,01%. We also included the two patient probes alone, each to define a basal SNP profile of the patients. This will also function as our negative control.
HID-Ion AmpliSeq Identity Panel shows 124 different SNPs of each patient.
First, we checked the frequency of the SNPs in the patients. The possible outcomes for a SNP are either homozygote, which means both alleles have the same nucleotide or heterozygote, which means that one allele represents a different nucleotide. The thresholds to believe a SNP is a homozygote one, is set for 0.14% of the reads at a lower level, and 0.75% of the reads at an upper level of the percentage from the total read fraction. In our mixture, there are 66 SNPs different between donor and recipient and 18 SNPs of these are homozygote and different. By looking at the frequency of the SNPs in the mixtures, in terms of total reads of one SNP, some nucleotides of the mixtures got more reads. There are nucleotides that are not represented in the donor, nor the recipient, or in the donor but not in the recipient and vice versa. In the mixed samples these nucleotides are also not represented. This may allow a precise evaluation as this SNPs could be used as

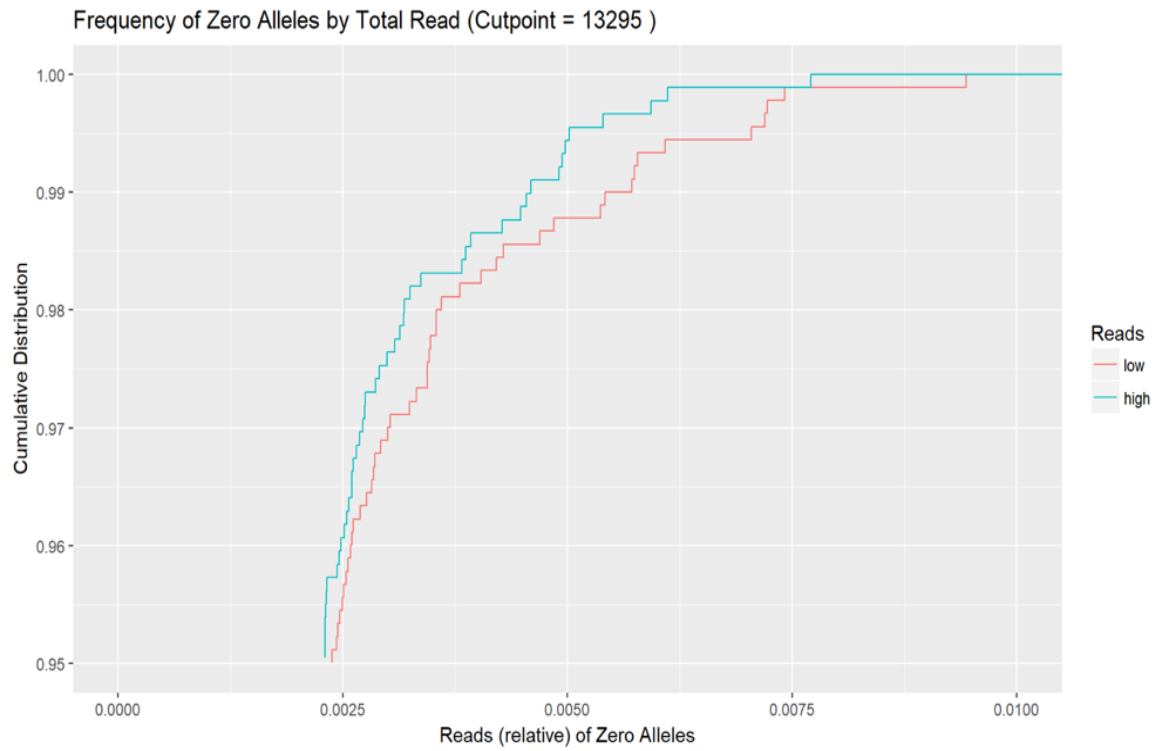


**Figure 5: The frequency of zero alleles by total reads is represented by the cumulative distribution of the SNPs and the reads by those alleles that are zero alleles. There is no significant difference by looking at the relative frequencies over 0.02. The reads above the 90% quantiles are possible candidates for the LOD calculation. (Graphic was reproduced with permission from Quehenberger F.)**

promising markers. The mixtures may also show some reads that are actually null reads. These are false-positive reads and increase with the number of total reads, but with increased total read counts there is a point when a plateau is reached. At this read count there is no significant difference by looking at the relative frequencies of these zero alleles above 0.02 cumulative distribution in low total reads and high total reads groups (Figure 5) (Wilcoxon-test: 0.28). The next step is to determine a distribution for the Limit of Detection (LOD), which is quantitated out of quantiles from small measured parameters e.g. the blank plus 3 times sigma. By using a non-parametric method of the relative abundance of the bases that should be zero it is possible to calculate the LOD (LOD = 0.014). A calculation with a parametric assumption resulted in a higher LOD (LOD = 0.018). The next question arises about the limit of detection and the according confidence interval. In EP17-A the Limit of Blank (LOB) is defined as the highest apparent analyte concentration at a 5% border. The LOD is the 5% of the measured points which are defined being under the LOB. The Limit of Quantification is the lowest concentration at which the cell-free DNA is reliably detected. Basically it is the bias plus 2 times the standard deviation, but always, at a minimum level, the LOD (127). The limit of detection is

the number of molecules that can be distinguished from a blank. This number of molecules is defined with the help of a standard curve. In this experiment our blank is represented by the null alleles which are known to be true negative. We included all SNPs to avoid a regression towards the mean bias. By only observing the relative allele count of homozygote and as well as heterozygote SNPs a Whiskers plot was drawn (Figure 6,7). Ever SNP value is independent from each other, because every SNP is an individual measurement due to individual primers. A Wilcoxon test and a Student´s t-test is therefore performed. A significant threshold of 0.05 has been set and the SNPs are divided into homozygote, heterozygote SNPs and grouped together.

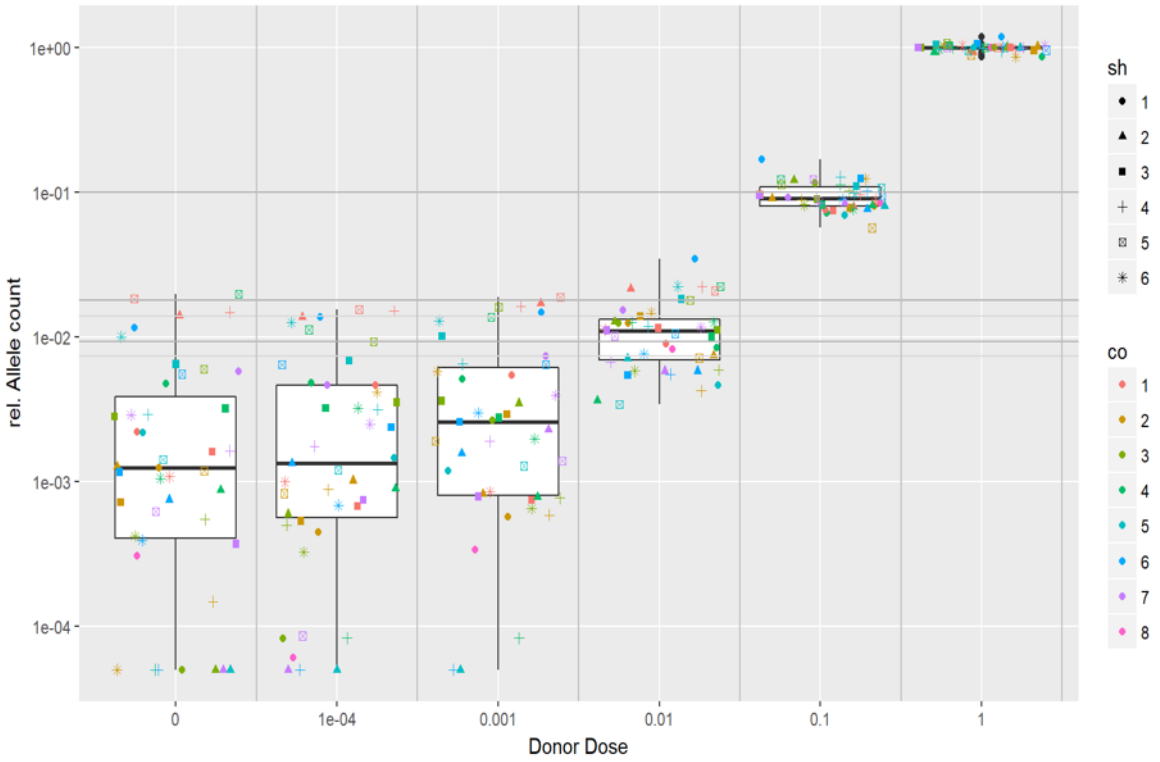


**Figure 6: Whiskers plot of homozygote + heterozygote SNPs. Every SNP is determined by a symbol. (Graphic was reproduced with permission from Quehenberger F.)**

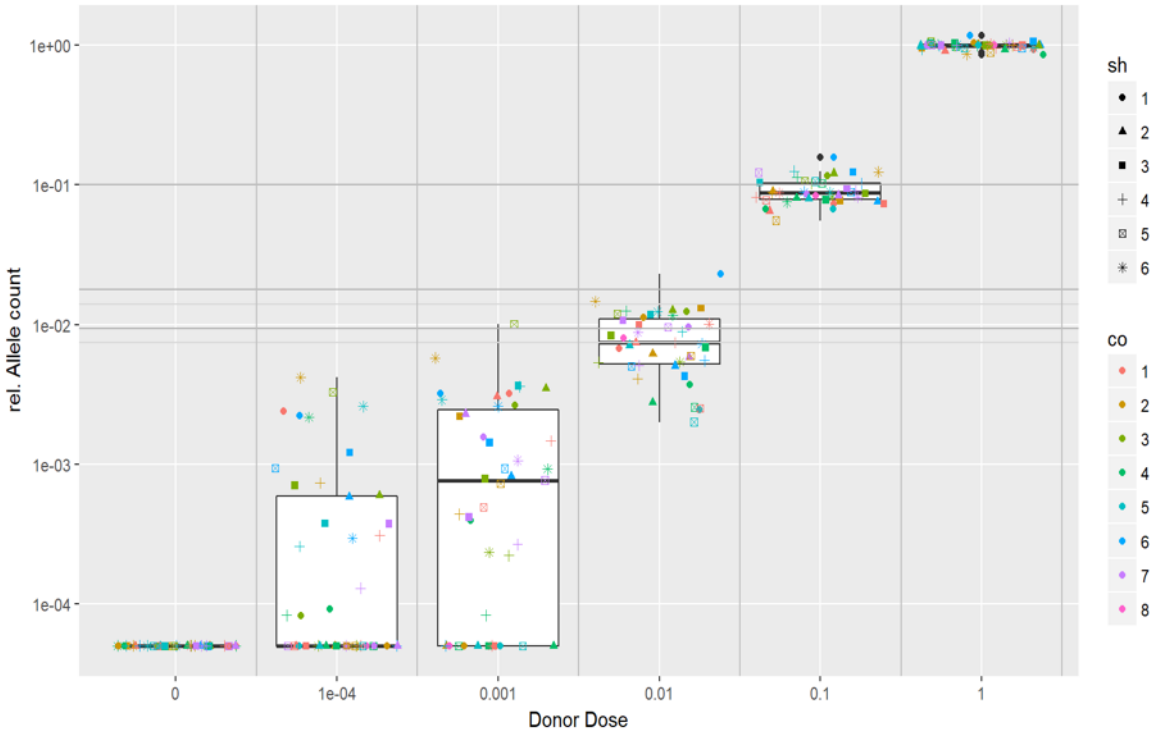


**Figure 7: Whiskers plot of homozygote + heterozygote SNP's. The blank value was subtracted. Every SNP is determined by a symbol. (Graphic was reproduced with permission from Quehenberger F.)**

A Fourth group was added named homozygote + heterozygote high abundance. The students t-test showed a significance level in comparison to a blank value with a p-value < 0.05 for the 100%, 10%, 1%, 0.1% group (Figure 8). The 0.01% showed a difference from the blank value which was not significant (p-value = 0.1248). The Wilcoxon signed ranks test showed the same results. Again, the group with 0.01% donor DNA could not be discriminated from the blank. The high abundance SNP's may induce a bias. Comparing the Wilcoxon signed rank test some values are statistical outliers (Figure 9). This value may skew the confidence interval boundaries because this is calculated as a quantile with all the possible SNPs measured. Question arises if we can improve our confidence interval by only using specific SNPs. Those outlying values then may be excluded from the calculation to reach an even lower LOD. The confidence interval itself can be calculated with all SNPs or by only using a group of SNPs which may improve the performance of the test. By further calculating a confidence interval for all the groups we found that using only specific SNPs did not improve our confidence interval. We concluded that working with all SNPs has the highest performance of the test.

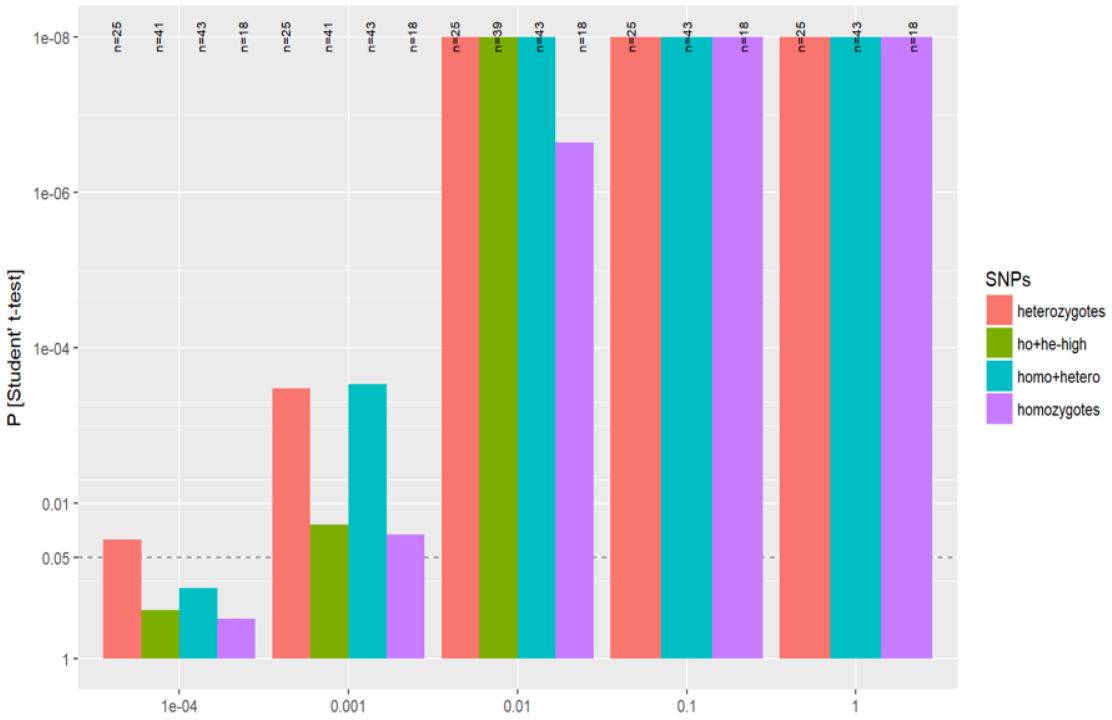


**Figure 8: Comparison from grouped SNP's to a blank value. p-values of Student's t-test are shown in the bars. (Graphic was reproduced with permission from Quehenberger F.)**

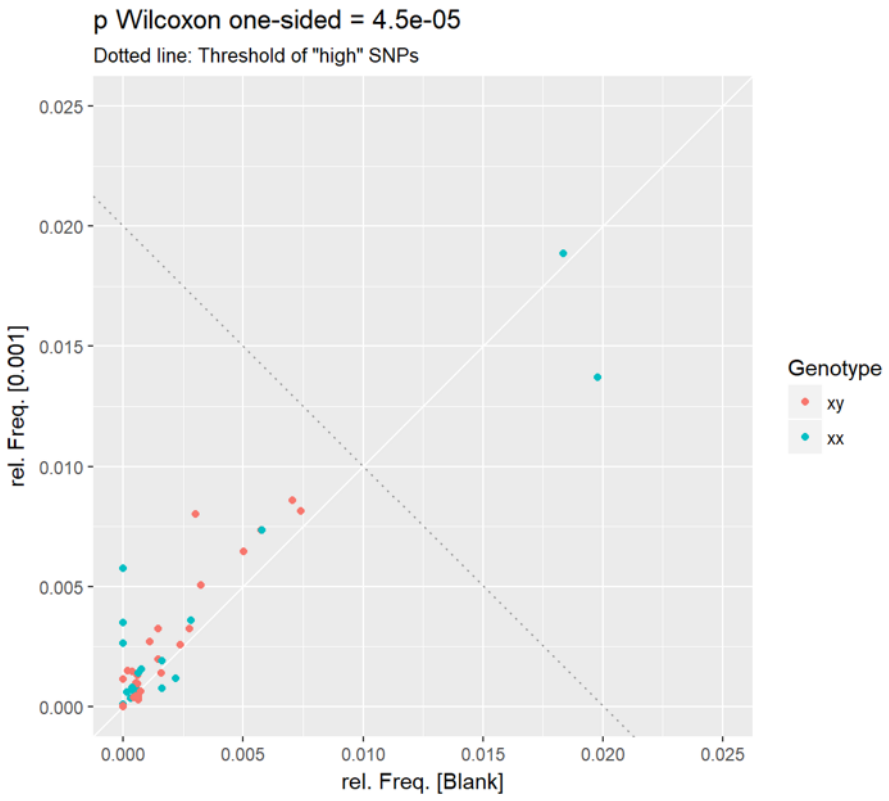


**Figure 9: The plot shows the Wilcoxon signed ranks test values of each SNP. Some SNPs are statistical outliers. Xx marks homozygote SNPs whereas xy marks the heterozygote ones. (Graphic was reproduced with permission from Quehenberger F.)**

The relative frequency of the base is separately used for all the counts of a dilution group. By using only homozygote or only heterozygote SNPs there is no improvement in performance. For further analysis we will use all SNPs (Figure 10).

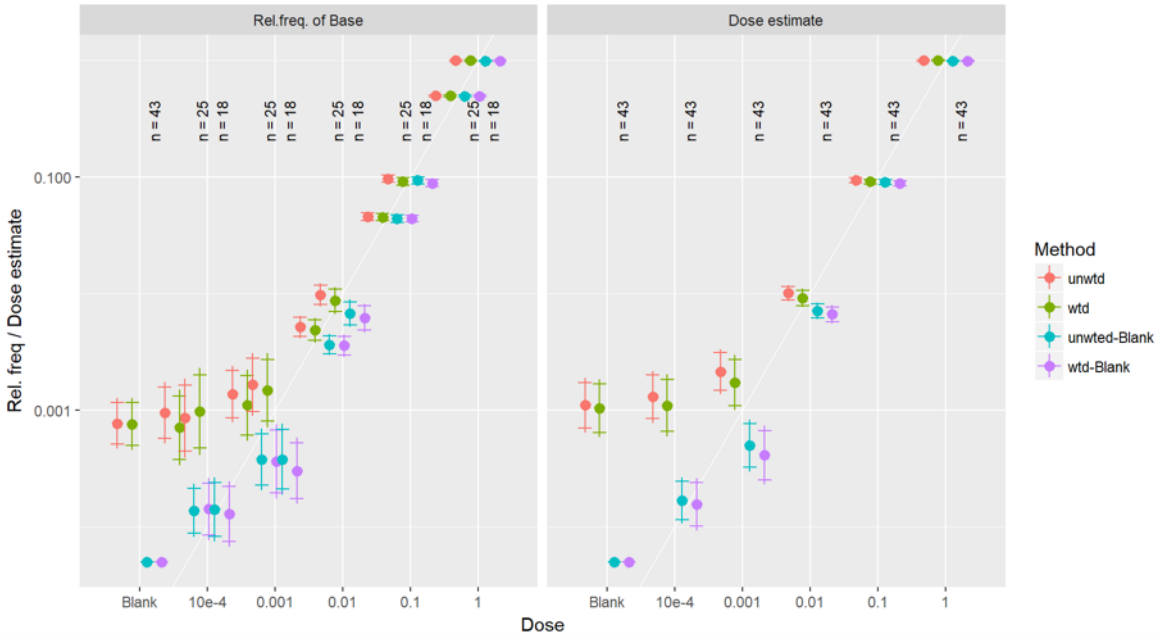


**Figure 10: Confidence boundaries are shown for homozygous and heterozygous SNPs (left) and for all the SNP counts together (right). The median and the 90% as well as the 95% confidence limits are shown in this plot. Wtd (weighted) and unwtd (unweighted) reads against the total reads are plotted. (Graphic was reproduced with permission from Quehenberger F.)**

## 3.2 MicroRNA profile

To conduct a biologically relevant answer to a microRNA profile, we have to compare our microRNA profile from liver and kidney tissue of transplant patients with healthy tissue. To accomplish this, we analyzed 3 healthy cryopreserved liver samples and 3 healthy cryopreserved kidney samples and performed a microRNA-sequencing to get a basal expression profile. Furthermore, we took two plasma samples from kidney transplant patients after transplant as a pilot-batch to compare the microRNA content of the plasma, with the basal expression of the originating tissue. After RNA isolation we measured the concentration. RNA concentrations are, by looking at the mean value, higher in liver than in kidney tissue which may represent a higher cell count in the liver (**Fehler! Verweisquelle konnte nicht gefunden werden.**). In the two plasma samples, the results were too low to be measured with a spectrophotometric method.

**Table 8: RNA concentration of cryopreserved tissues after RNA isolation.**

| Liver (RNA concentration) | | | Kidney (RNA concentration) | | |
|---|---|---|---|---|---|
| Liver 1 | Liver 2 | Liver 3 | Kidney 1 | Kidney 2 | Kidney 3 |
| 1460ng/µl | 2425,2 ng/µl | 1521 ng/µl | 500 ng/µl | 666,9 ng/µl | 846,4 ng/µl |

The microRNA fraction is more robust to degradation due to their smaller length, nevertheless we checked for RNA quality. The RNA Integrity number (RIN) was higher in the liver tissue (Figure 11). The median RIN of liver samples was 4.8 (Sample 1= 5.9 Sample 2= 3.8 Sample 3= 4.9) compared to kidney specimens where the median RIN was 2.7 (Sample 4= 2.8 Sample 5= 2 .1 Sample 6= 3.2). After generating the library, we checked again for RNA quality and RNA fractions to measure the amount of microRNA (Figure 12). The microRNA peak should be at approximately 107bp - after adapter and barcode ligation. Again, higher median microRNA amounts were found in the liver tissues (liver = 2547 pg/µl, kidney = 3111 pg/µl) (Table 9).

**Table 9: MicroRNA concentration from the Bioanalyzer chip. The concentration above is measured and calculated as the area under the peak at 107bp.**

| Liver (microRNA concentration) | | | Kidney (microRNA concentration) | | |
|---|---|---|---|---|---|
| Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 2506 pg/µl | 2505 pg/µl | 2630 pg/µl | 868 pg/µl | 842 pg/µl | 1401 pg/µl |

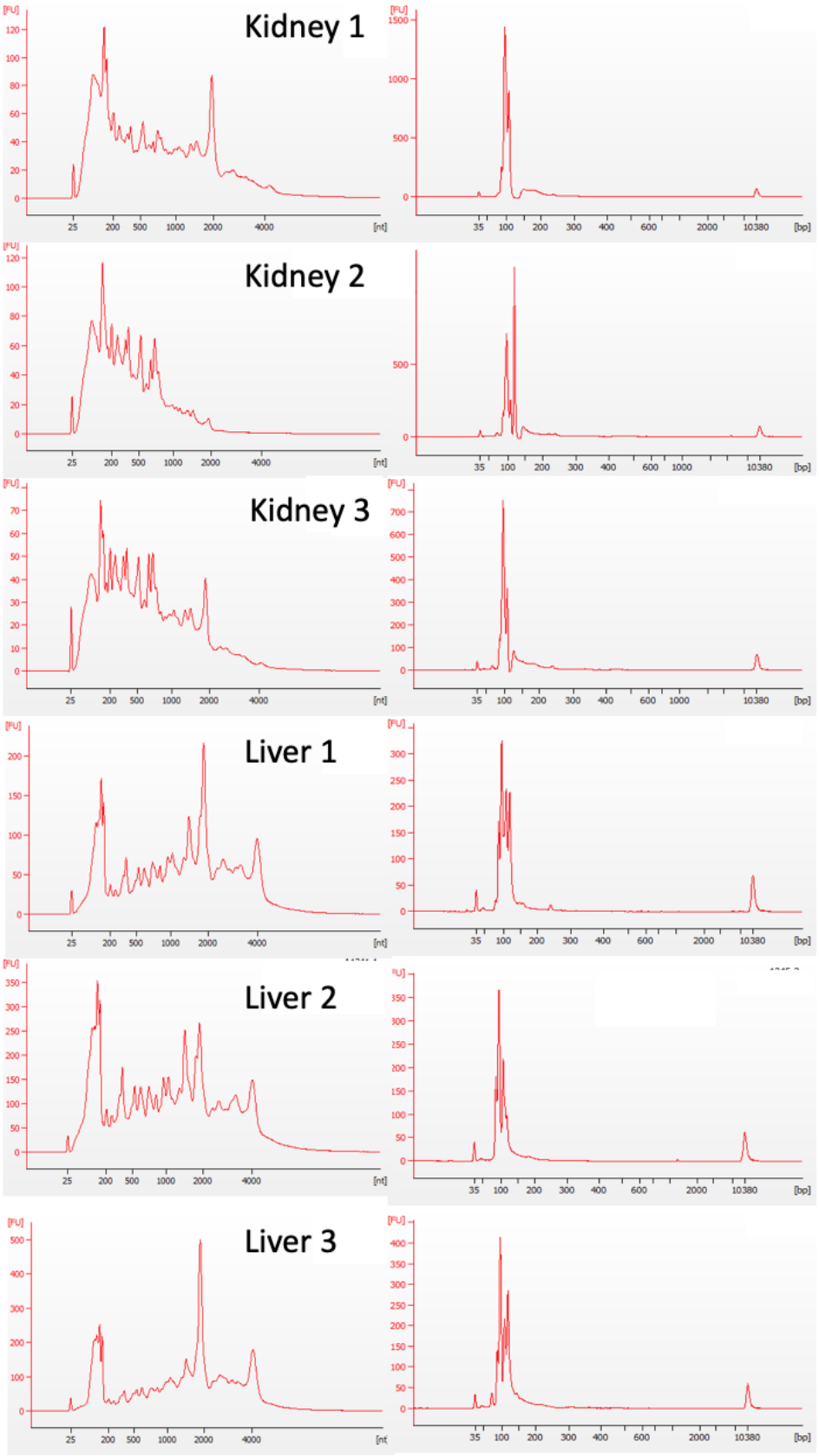


**Figure 11: The microRNA fractions are presented on a 2-dimensional graphic. The peaks are representing the highest FU which represents the concentration for the specific fragment length. Left plots are representing the RNA before library creation, right plots are showing the final library.**

We then sequenced and analyzed the data through our bioinformatic pipeline (Table 10). The normalized read counts are further used and processed for a differential expression analysis.

**Table 10: MicroRNA reads after sequencing. According to the CAP-miRSeq V1.1 the reads are processed and shown in this sample summary (125).**

| Sample | Total Reads | Trimmed Reads | Reads sent to Aligner | Aligned Reads | Precursor miRNA Reads | Mature miRNA Reads | Known miRNA with >= 5x coverage |
|---|---|---|---|---|---|---|---|
| Liver 1 | 8,80E+06 | 3,13E+05 | 3,47E+06 | 2,68E+06 | 3,46E+03 | 1,57E+06 | 5,75E+02 |
| Liver 2 | 1,01E+07 | 2,07E+05 | 6,51E+06 | 2,14E+06 | 2,43E+03 | 9,46E+05 | 5,26E+02 |
| Liver 3 | 1,17E+07 | 3,88E+05 | 4,76E+06 | 3,51E+06 | 4,19E+03 | 2,07E+06 | 5,97E+02 |
| Kidney 1 | 1,01E+07 | 2,07E+05 | 5,06E+06 | 2,90E+06 | 3,01E+03 | 1,61E+06 | 6,18E+02 |
| Kidney 2 | 1,07E+07 | 2,19E+05 | 5,35E+06 | 2,50E+06 | 2,32E+03 | 1,21E+06 | 5,79E+02 |
| Kidney 3 | 1,08E+07 | 3,39E+05 | 3,95E+06 | 3,03E+06 | 2,68E+03 | 2,25E+06 | 6,62E+02 |
| Plasma 1 | 4,42E+05 | 6,65E+03 | 1,27E+05 | 5,53E+03 | 1,10E+01 | 2,77E+02 | 1,10E+01 |
| Plasma 2 | 3,16E+05 | 6,20E+03 | 2,16E+05 | 1,03E+04 | 4,00E+00 | 1,97E+03 | 6,40E+01 |

First, we normalized the reads and used the counts for a principal component analysis (PCA). Graphically shown: The healthy liver tissue and healthy kidney tissue samples are clustered together in this analysis. Also, the plasma samples are shown to have lower variances. X and Y axis shows principal component 1 and principal component 2 that explains 50.4% and 26.7% of the total variance with our 8 datapoints. Furthermore, we created a heatmap to visualize our differentially expressed miRNAs (Figure 12). Again, liver and kidney clustered together in a tree. Interestingly also our plasma samples of kidney transplant patients showed a higher clustering to the kidney branch than to the liver branch (Figure 13). This could be explained by a higher count of kidney specific microRNAs in the plasma after transplantation due to organ damage.

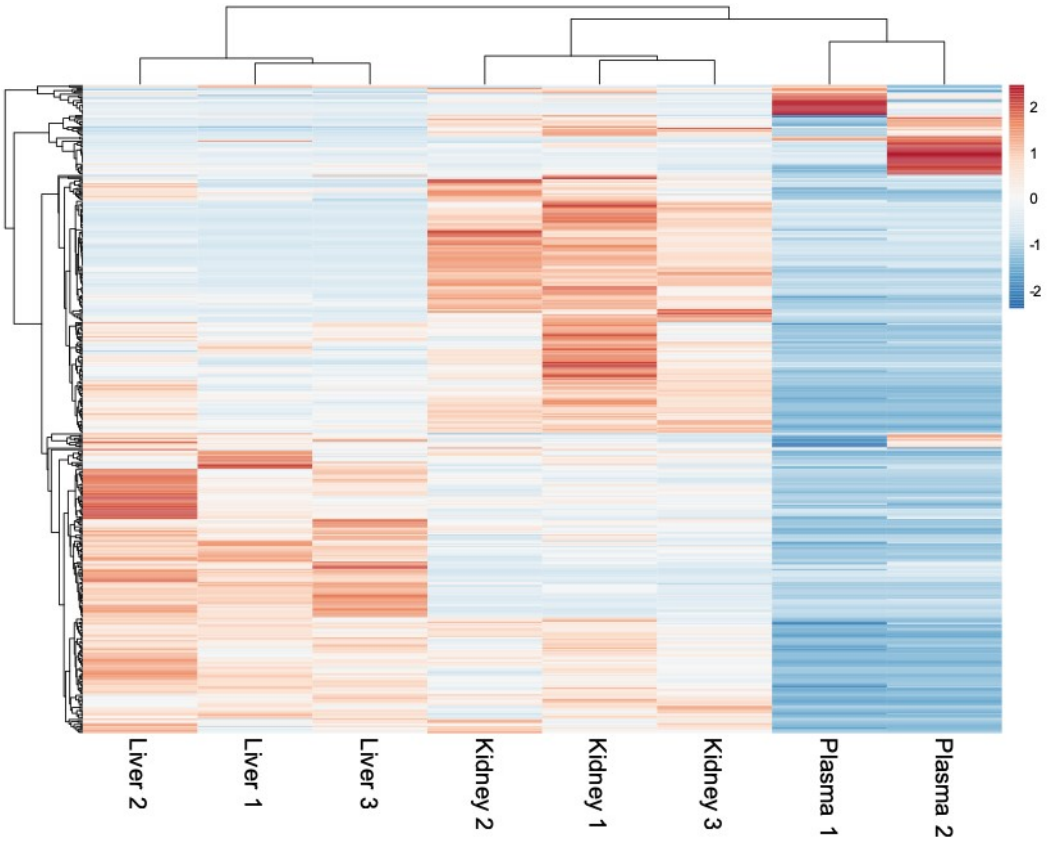


**Figure 12: Normalized microRNA counts are presented in a heatmap. Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using correlation distance and average linkage. 454 rows, 8 columns (130).**

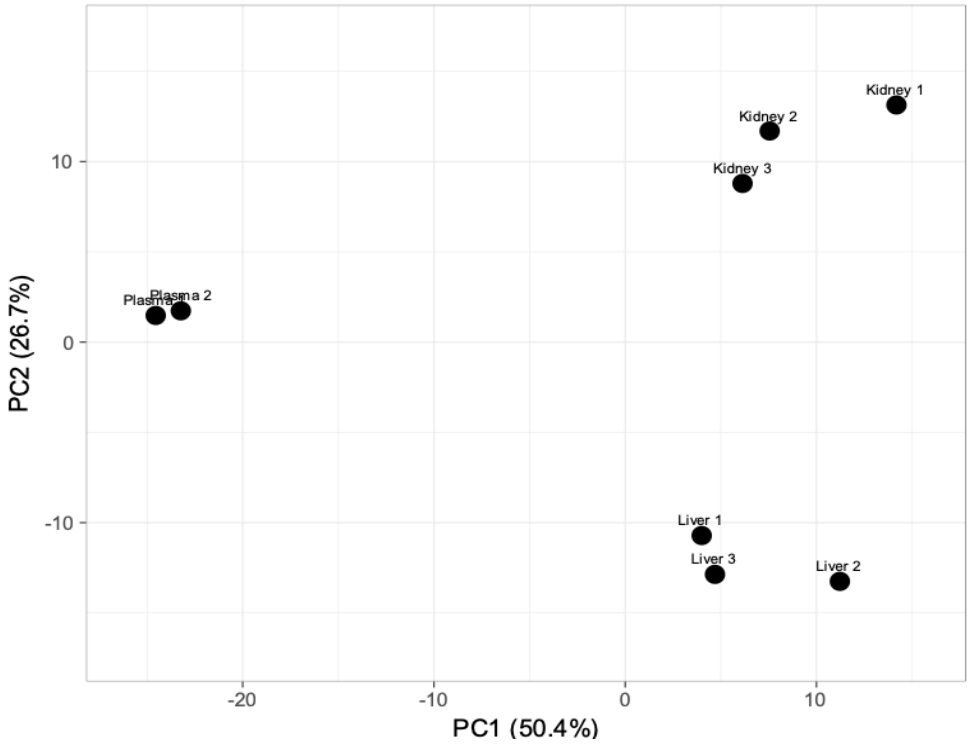


**Figure 13: Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 50.4% and 26.7% of the total variance, respectively. N = 8 data points (130).**

By comparing the amount of microRNA with the concentration after the RNA isolation there is no significant relationship (p= >0.05) between the tissue samples with a higher concentration or a higher microRNA count. By looking at tissue-specific expressed microRNAs and comparing them with tissue specific miRNAs, it is possible to depict specific microRNAs, for example miR10b-5p or miR204-5p are kidney-specific and are expressed in kidney tissues and not in the liver samples (128). Vice versa miR-122-5p and miR-122-3p are more liver specific and expression profile also showed high expression liver samples and no expression in the kidney ones (128). The highest abundance in expression counts for the plasma samples is shown for miR-223-3p, miR-19b-3p, miR-451a and miR-122-5p. Those microRNAs are widely distributed across tissues but are mostly expressed in veins and vessels: The high counts could be due to a bias of the venipuncture. Interestingly, miR-223-3p is also related to transplant rejection in the literature (129).

## *3.3 T-cell receptor sequencing*

### 3.3.1 Proof of concept

At first, we wanted to establish the TCRB sequencing by comparing whole blood and sorted fractions in order to do a proof of concept. Therefore, we started with 500k FACS sorted CD4+-T-cells and whole blood samples from kidney transplant patients and isolated DNA. For our first approach we used 5 ng DNA input for the multiplex PCR. Sequencing results showed higher counts of unique CDR3 sequences in the sorted T-cell fraction n=1877 in comparison to the whole blood sample n=71. This could be explained due to the fact that in whole blood samples only 10%-20% of the mononuclear cells represent cell types, that are expressing a T-cell receptor beta which consists the CDR3 region. We further saw that the unique CDR3 regions of our samples showed – despite the fact that both are from the same patient – a high sampling bias. Only six CDR3 regions had an overlap between both fractions. This could be answered by the fact that in our blood stream millions of leukocytes circulate and with 5ng of DNA input only a small fraction is accessible for sequencing.

### 3.3.2 Optimization and Sampling Bias

The next step was to explore and circumvent the sampling bias with more input DNA and to compare 2 methodic approaches. Following a basic arithmetic calculation: DNA has 650 (g/mol)/bp with a length of 3,2MBp in *homo sapiens* that leads to 14500 cells in 50ng DNA. Assuming a 10-20% fraction of lymphocytes the input of 50ng DNA results in approximately 2000-3000 genome equivalents (GE). 1ml of blood consists of circa 30million leukocytes which are again 3-6 million lymphocytes. We extracted DNA from 300 µl of whole blood. Extraction yielded approx. 120 ng/µl which are 12 µg total DNA. This means by just adding 50ng of DNA (which is a limit in some PCR manuals), it yields only a small fraction of the whole abundance (50ng is equivalent to 10000 cells out of 8 million per ml blood which is only). The next step was to prove this theoretical construct in vitro.

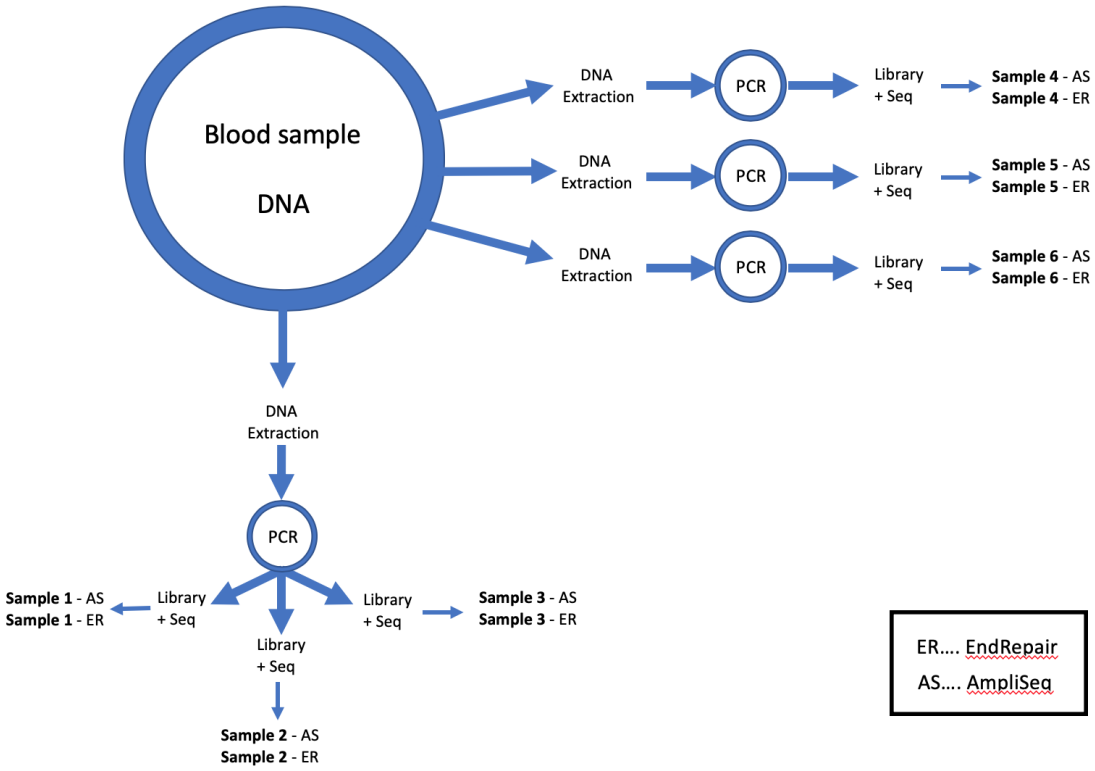


**Figure 13: Schematic drawing of the workflow in our optimization and Sampling Bias experiment. In sample 1- 3 one multiplex PCR was performed with a total of 50ng DNA. For the samples 4-6 we used 50ng for each multiplex PCR to assess the diversity of the TCRB pool.**

We compared two different methods for the library preparation: The AmpliSeq and a universal End-Repair approach. For both methods we examined more deeply

the before mentioned sampling bias. To do this, we divided the two methods further into two groups. In the first group 50 ng of DNA is used for the multiplex PCR out of the isolated DNA and is further processed. In the second group we used 50ng for every sample for the PCR again out of the same DNA tube (Figure 13). This means that within the group 1 we should see a larger overlap of CDR3 regions then in group 2 -if a sampling bias exists- where we can evaluate the confounding factor. Furthermore, it is also possible to compare AS with ER.

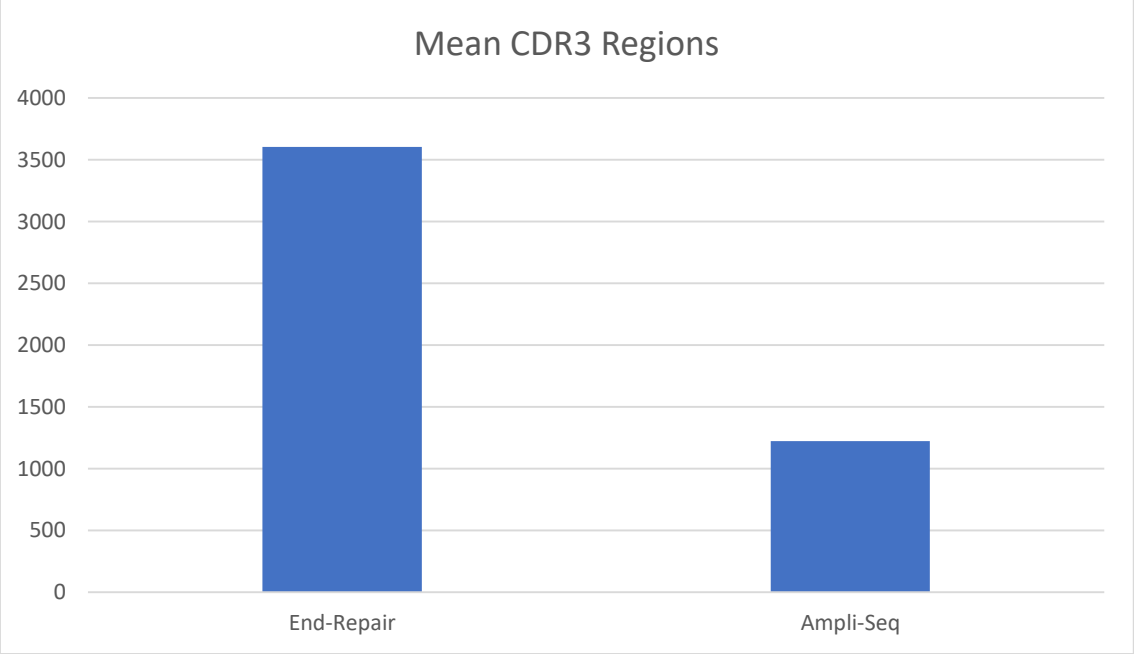


**Figure 14: In this figure the comparison of unique CDR3 regions at amino acid levels shows a higher count with the End-repair method. All samples described in Figure 14 are added and a mean value is calculated.**

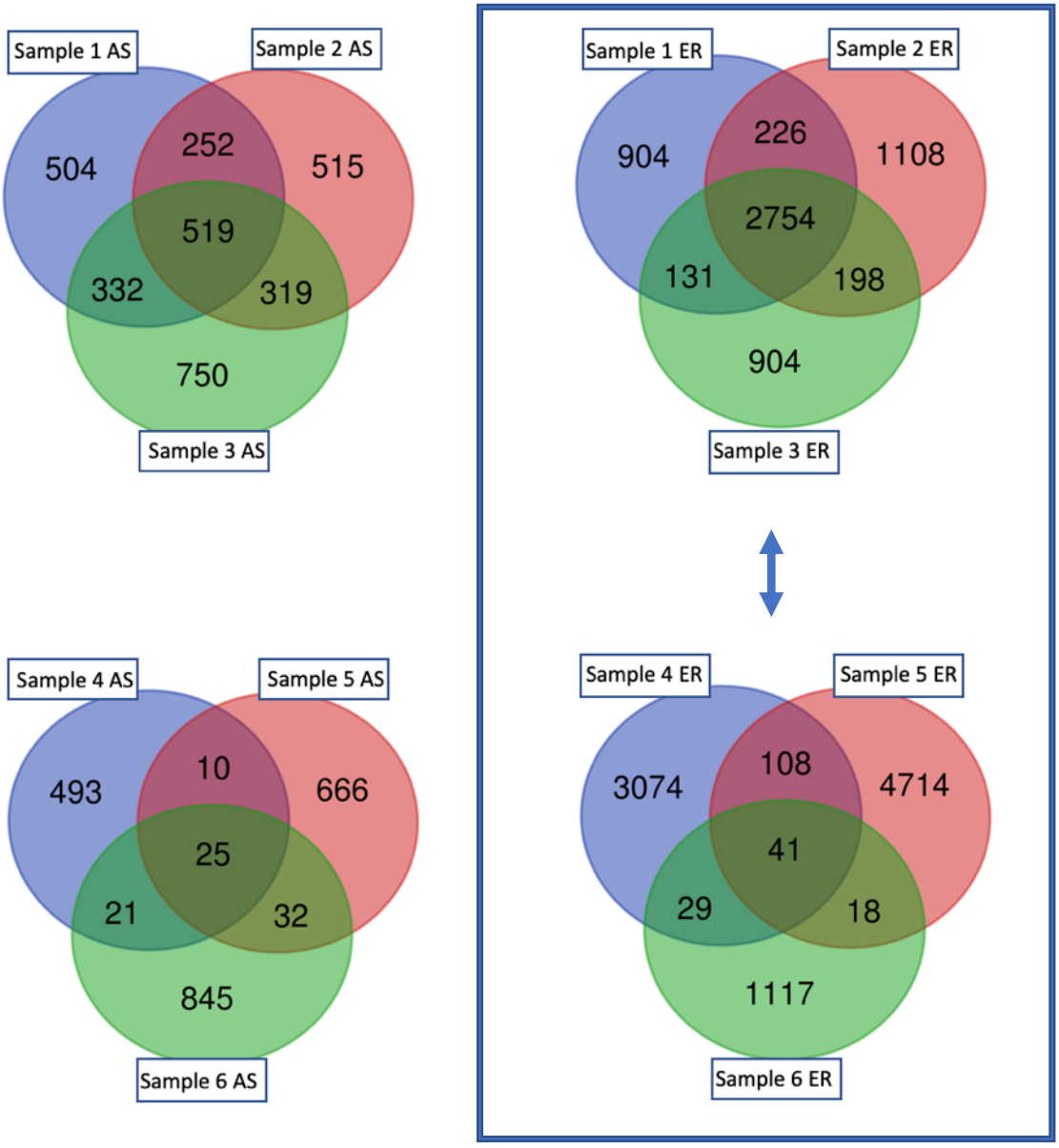


**Figure 15: This Venn diagrams shows an overlap of unique CDR3 regions at amino acid level of the different methodic approaches. 6 Samples are performed with an End-repair approach, the other six with the AmpliSeq. The unique circles represent one library preparation. Samples 1,2,3 are processed with the same multiplex PCR whereas Sample 4,5,6 got different DNA samples as input. The highest overlap is seen in the End-Repair samples with the same multiplex PCR DNA pool.**

First, the overall mean amount of unique CDR3 regions was way higher with the universal end-repair approach (Figure 14). Secondly, by comparing the overlapping CDR3 regions of three samples, processed with the same method out of the same blood tube, there is a higher overlap by using the same DNA for downstream sequencing than by using 3 individual samples of the same blood tube (Figure 15). This kind of proved the sampling bias that appeared in our first approach. What is

also interesting, is that in sample 4,5,6 from both AmpliSeq (AS) and End-repair (ER) the overlapping clones are also represented in the top 10 tiers of the CDR3 regions, sorted by abundance, between all three samples. This means that even if there are millions of T-cell clones and unique CDR3 regions, the most prominent - which may be due to clonal expansion - can be found more often or even in every sample. The CDR3 region with the highest abundance was found in Sample 4 ER with 4% of the total counts.

The Shannon's diversity index is another indicator used in TCRB-Sequencing. It represents the abundance of an individual in a whole species or family. By comparing this index with the different methods, we found no significant difference between the End-Repair and the AmpliSeq methods or within the method (Figure 16). The Shannon Index showed a high comparability between the triplicates which kind of proved our method. Furthermore, the ER approach seemed to depict a higher diversity which may be nearer to the true diversity on the sample.

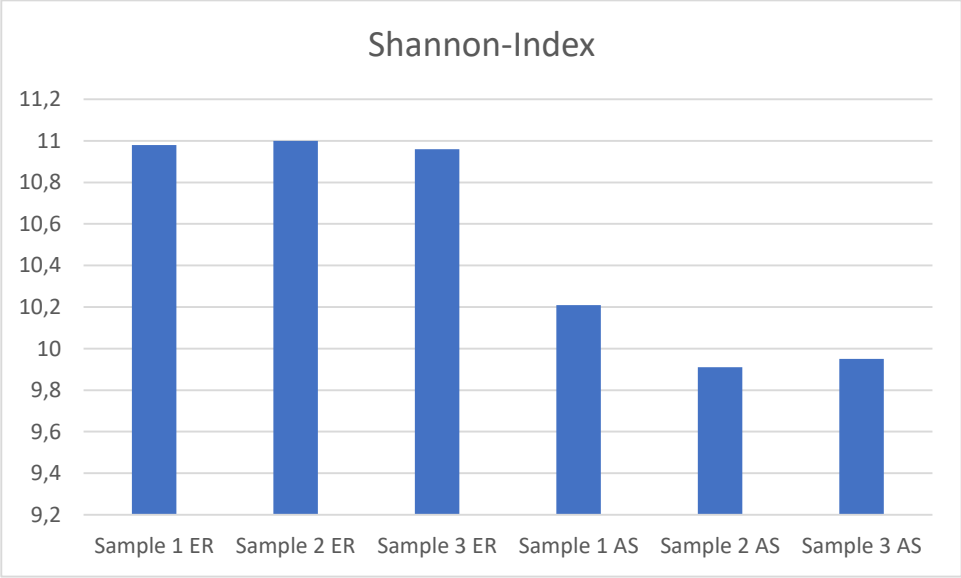


**Figure 16: This bar diagram shows the values of the Shannon indices from different samples. The same probes were also used before for the Venn diagram. Shannon-Index was calculated within the bioinformatics pipeline (1)**

### 3.3.3 PCR optimization

For the optimal PCR annealing temperature, we further compared the same DNA with either 60°C and 65°C. The sample with the 60°C PCR got a unique number of CDR3 regions of 1938 whereas the 65°C sample yielded 8341. The Raw Sequence

Number served as an internal control. Interestingly the effectively mapped reads – reads that align to VDJ regions – increased as well as the before mentioned CDR3 regions (Figure 17) for the 65°C PCR. This means that the overall PCR is more specific for the VDJ regions. By comparing the Shannon index, the number is not significantly different between the two samples - Shannon index 8.85 and 8.94 ($p>0.05$).

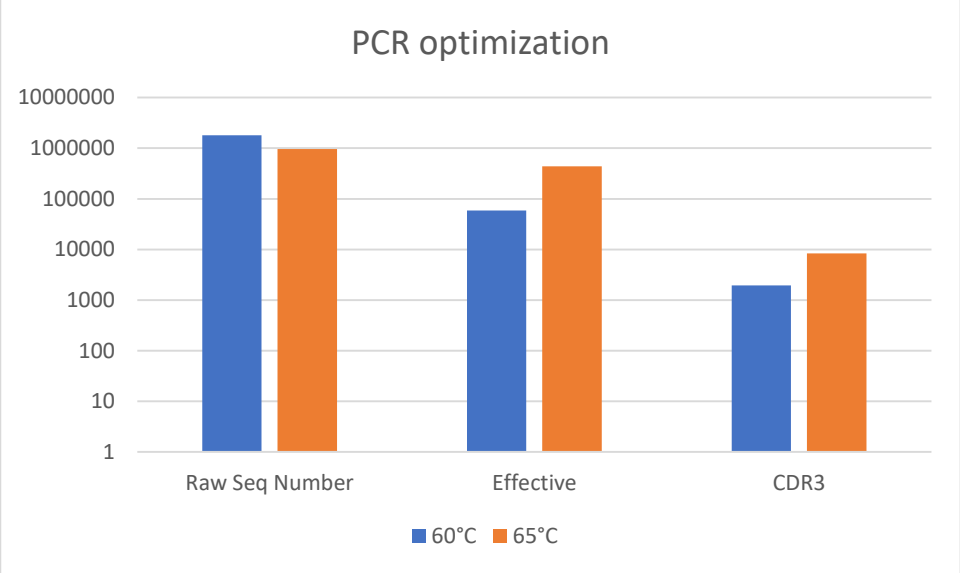


**Figure 17: Unique CDR3 regions are shown in this bar diagram. Multiplex PCR was either done with 60°C or 65°C. Raw Sequence Number, Effective Reads that aligned to VDJ as well as unique CDR3 regions are shown in a bar graph.**

Changing the annealing temperature also varied V(D)J usage. Some primers may enhance their binding affinity which resulted in different V(D)J usage patterns.
The pipeline also consists of graphical output from diverse indicators that may help to interprete the data. The CDR3 got a median length of 44 nucleotides on average. This spectrum consists of V, D and J regions which differ in length, due to usage of the specific regions after recombination. Measuring of hypermutation is also possible and can be calculated by aligning the sequence with the genomic regions of a reference genome within the regions itself. Hypermutation of this sample, which is the optimized 65°C sample, shows a 1% sequence mutation rate for the V region, 0% for the D and 0.39% for the J region - that results in an overall base mutation rate of 1.2% (Figure 18).

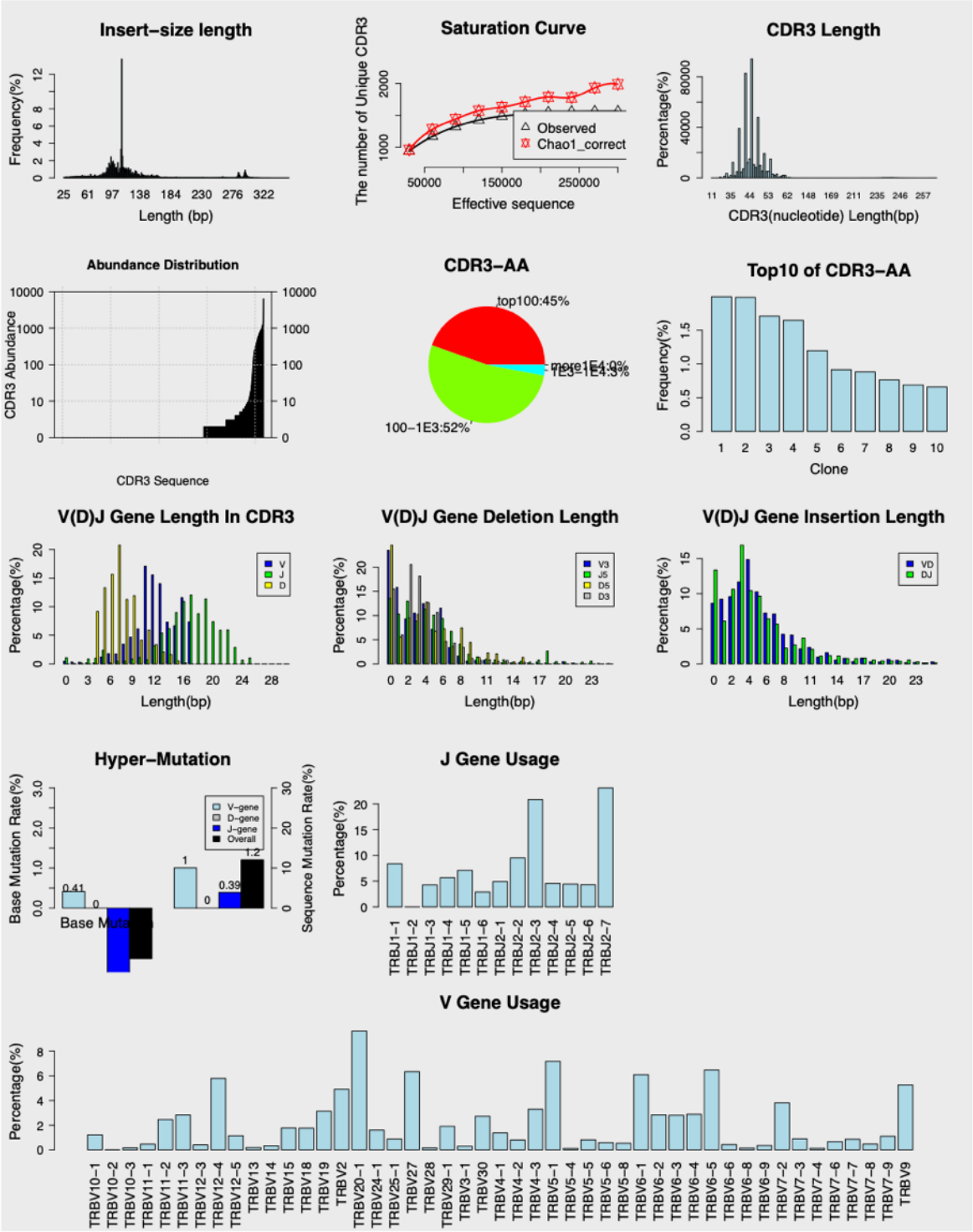


**Figure 18: The 65°C sample was used for a comprehensive analysis through our pipeline. Shown from top to bottom and left to right: Insert-size length and their respective frequency; the Saturation curve indicated the bias introduced by the multiplex PCR. CDR3 region length and abundance distribution; the circle plot shows the distribution of the top 100 and top 1000 clones. The top 10 clones are shown in a bar graph. V(D)J distribution; in respect to CDR3 region length, deletion length and insertion length is also represented; hypermutation status shows mutations that differ from the genomic V(D)J regions and is presented as percentage.**

# 4 Discussion

The SNP profiling of cell-free DNA is basically a form of liquid biopsy. The term is normally reserved for tumour diagnostics but lately it has been used for general medical questions. By taking advantage of the preexisting knowledge of this method, we used liquid biopsy to detect transplant rejection, by analyzing nucleic acids. Firstly, we started with the SNP profile, that is unique in every human, by including all of the polymorphisms in the whole genome. Due to cost and efficiency, the goal is to reduce the number of SNPs to a basic profile, which leads to discrimination in a high percentage of human individuals. For this approach we used the SNPs in the Human Identitiy Panel, which is basically used in forensics to classify unknown DNA. This original use benefits our scientific question. At its best, it should also be possible to distinguish even siblings, because renal transplantation is often carried out between relatives. Furthermore, the cell-free DNA itself is only present in small fractions within the blood stream. So to reliably detect the cell-free DNA, which is shed by the donor in the recipients blood stream, we have to set a limit at which we believe the value called limit of detection. The dilution experiment determined a limit of detection at 0.01 allelic fraction by including at least 44 SNPs of the whole 124 SNP pool. This limit is variable due to the number of distinct SNPs. Patients are discriminated by using unique SNPs between donor and recipient, we may further reduce the limit or may lose sensitivity with less distinct SNPs. Results showed that it is better to use all of the SNPs in the analysis and to not reduce the panel. These resulted in a smaller confidence interval. Furthermore after detection once a SNP profile of distinct SNPs, we can set up a personalized and specific test for each patient. We may use only a smaller number of unique SNPs to trackthe transplant rejection in this patient, with other, more sensitive methods, like digital droplet PCR (ddPCR).

The microRNA approach showed the feasibility of detecting novel or known biormarkers involved in transplant rejection. Due to the specificity of organ damage of the transplanted organ, we can correlate this with a microRNA profile. In our small pilot cohort we have shown that microRNAs, that are highly abundant, are organ specific, but there are also some pan-miRNAs which may indicate a immune response. Nevertheless microRNAs are a diverse fraction and a reliable analysis should include every highly abundant one that is then further interpreted in relation to its biological known function and tissue origin. The blood plasma fraction only

contains a small amount of microRNAs, that may be biased due to the venipuncture. We saw highly abundant microRNAs in the plasma, that are associated with vessels or venes. But being aware of this confounding factor the miRNA profile may be another promising biomarker for organ rejection detection.

The third part of our three blood based biomarkers is measured within the T-cell population. T-cells are prone to attack infected and foreign cells, due to their specificity for presented antigens. The T-cell receptors most specific region – the CDR3 region - represents the population and therefore a expansion of the CDR3 region may lead to the conclusion, that the T-cells have clonally expanded against the organ. This clonal expansion is part of the immune response, in terms of transplantation this expansion should be suppressed. Sometimes this suppression fails or is insufficient and T-cells undergo a clonal expansion, that leads to organ destruction and graft rejection. We used a DNA approach to detect the CDR3 region which is in comparision to a RNA approach more feasible and it lacks the RNA expression bias. The sampling bias is problematic if one specific clone, that is present at a small abundance is targeted for search - but if a T-cell undergoes clonal expansion, the risk for missing this clones is way less. It is also possible to track specific clones that have expanded in prior immune rejections or however the immune system is challenged. In this case the sequence is known and it is possible to track these clones and see the recapitulating expansion or the decrease, which then may lead to varying immunosuppressive therapy. The Shannon-Index may be a marker for this clonal expansion. In a immune reaction some clones get a high abundance and the shannon index will change. This change in the index number can indicate a T-cell mediated rejection. On its own, the clonal expansion of a T-cell population is not a strong marker because it lacks of sensitivity, but with the combined use of the other biomarkers mentioned we may give a more precise answer to this question.All in all this pilot study showed the feasbility and robustness at a methodical and a biological level of all three biomarkers for transplant rejection. In order to gain statstic relevant answers to our next question - which biomarker is the most promising - we have to collect a higher number of samples from patients that undergo transplant rejection. This may ultimately reveal the applicability of our biomarkers. Cell-free DNA indicates the organ damage, microRNA may show the underlying pathophysiologcal function, and the TCRB analysis could highlight an autoimmune cause against the organ due to a immunological attack of T-cells.

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# Anhang – Multiplex Primer Liste

| Region | Primer Sequence |
|---|---|
| TRBJ1.1 | CTTACCTACAACTGTGAGTCTGGTG |
| TRBJ1.2 | CTTACCTACAACGGTTAACCTGGTC |
| TRBJ1.3 | CTTACCTACAACAGTGAGCCAACTT |
| TRBJ1.4 | AAGACAGAGAGCTGGGTTCCACT |
| TRBJ1.5 | CTTACCTAGGATGGAGAGTCGAGTC |
| TRBJ1.6 | CATACCTGTCACAGTGAGCCTG |
| TRBJ2.1 | CCTTCTTACCTAGCACGGTGA |
| TRBJ2.2 | CTTACCCAGTACGGTCAGCCT |
| TRBJ2.3 | CCGCTTACCGAGCACTGTCAG |
| TRBJ2.4 | AGCACTGAGAGCCGGGTCC |
| TRBJ2.5 | CGAGCACCAGGAGCCGCGT |
| TRBJ2.6 | CTCGCCCAGCACGGTCAGCCT |
| TRBJ2.7 | CTTACCTGTGACCGTGAGCCTG |
| TRBV2 | ATTTCACTCTGAAGATCCGGTCCAC |
| TRBV3-1 | AAACAGTTCCAAATCGMTTCTCAC |
| TRBV4-1.2.3 | CAAGTCGCTTCTCACCTGAATG |
| TRBV5-1 | GCCAGTTCTCTAACTCTCGCTCT |
| TRBV5-4.5.6.8 | TCAGGTCGCCAGTTCCCTAAYTAT |
| TRBV6-4.1 | CACGTTGGCGTCTGCTGTACCCT |
| TRBV6-8.5.1.2 | CAGGCTGGTGTCGGCTGCTCCCT |
| TRBV6-9.7.1.1.6 | CAGGCTGGAGTCAGCTGCTCCCT |
| TRBV6-4.2 | AGTCGCTTGCTGTACCCTCTCAG |
| TRRBV6-2.3 | GGGGTTGGAGTCGGCTGCTCCCT |
| TRBV7-2.4.6.7.8 | GGGATCCGTCTCCACTCTGAMGAT |
| TRBV7-3 | GGGATCCGTCTCTACTCTGAAGAT |
| TRBV7-9 | GGGATCTTTCTCCACCTTGGAGAT |
| TRBV9 | CCTGACTTGCACTCTGAACTAAACCT |
| TRBV10-1 | CCTCACTCTGGAGTCTGCTGCC |
| TRBV10-2.3 | CCTCACTCTGGAGTCMGCTACC |
| TRBV11-1.2.3 | GCAGAGAGGCTCAAAGGAGTAGACT |
| TRBV12-3.2.5.2 | GAAGGTGCAGCCTGCAGAACCCAG |
| TRBV12-3.1.4.5.1 | GAAGATCCAGCCCTCAGAACCCAG |
| TRBV13 | TCGATTCTCAGCTCAACAGTTC |
| TRBV14 | GGAGGGACGTATTCTACTCTGAAGG |
| TRBV15 | TTCTTGACATCCGCTCACCAGG |
| TRBV16 | CTGTAGCCTTGAGATCCAGGCTACGA |
| TRBV18 | TAGATGAGTCAGGAATGCCAAAG |
| TRBV19 | TCCTTTCCTCTCACTGTGACATCGG |
| TRBV20-1 | AACCATGCAAGCCTGACCTT |
| TRBV24-1 | CTCCCTGTCCCTAGAGTCTGCCAT |
| TRBV25-1 | GCCCTCACATACCTCTCAGTACCTC |
| TRBV27-1 | GATCCTGGAGTCGCCCAGC |
| TRBV28 | ATTCTGGAGTCCGCCAGC |
| TRBV29-1 | AACTCTGACTGTGAGCAACATGAG |

**Table 11 : Primers were obtained by the IMGT database (131,1). These primers are used for the multiplex PCR for TCRB sequencing.**

# Anhang – Informed consent



**PatientInneninformation[1] und Einwilligungserklärung für Erwachsene zur Teilnahme an der Studie:**

**Vergleichende molekulare[2] Analysen von zirkulierender[3] DNA/microRNA und Immunzellrezeptor-Repertoire[4] als Biomarker[5] für Gewebsschäden und Transplantatabstoßung**

Sehr geehrte Teilnehmerin, sehr geehrter Teilnehmer!

Wir laden Sie ein an der oben genannten medizinischen Studie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

**Ihre Teilnahme an dieser Studie erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.**

Diese Studien sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer solchen Studie ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser Studie schriftlich erklären. Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Arzt sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur:

- wenn Sie Art und Ablauf der Studie vollständig verstanden haben,
- wenn Sie bereit sind, der Teilnahme zuzustimmen und
- wenn Sie sich über Ihre Rechte als Teilnehmer an dieser Studie im Klaren sind.

[1]Wegen der besseren Lesbarkeit wird im weiteren Text zum Teil auf die gleichzeitige Verwendung weiblicher und männlicher Personenbegriffe verzichtet. Gemeint und angesprochen sind – sofern zutreffend – immer beide Geschlechter.

[2]Makromoleküle der Biologie betreffend

[3] Im Blut im Umlauf sein

[4] Bestand an verschiedenen, dem Immunsystem zugehörigen Zellen, insbesondere deren Zellrezeptoren

[5] empirisch messbarer Wert im Bereich der Biomedizin



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Zu dieser Studie, sowie zur Patienteninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

## 1. Was ist der Zweck der Studie?

In Europa leben 250.000 Menschen mit einem Spenderorgan. In Zukunft wird diese Zahl noch weiter steigen da chronische Erkrankungen, durch unsere alternde Gesellschaft, weiter zunehmen werden. Im Gegensatz dazu steht ein Mangel an zu transplantierenden Organen und damit die äußerste Sorgfalt der Ärzteschaft diese Organtransplantationen risikoarm handzuhaben.

Transplantierte Organe stellen für den Organismus fremdes Material dar. Unser Immunsystem erkennt dieses und startet mit einer Abwehrreaktion – Darauf kann im schlimmsten Fall eine Transplantatabstoßung gipfeln.

Um das zu verhindern hat die Früherkennung von Abstoßungsreaktionen eine entscheidende Bedeutung. Als zuverlässigste Methode gilt die Biopsie. Diese Methode ist jedoch mit einem Risiko für Komplikationen verbunden und somit wird intensiv an Methoden geforscht, welche diese Risiken minimieren können. Eine davon ist die Suche nach Markern im Blut. Dadurch könnte man die Biopsie mit einer Blutabnahme ergänzen um die Treffsicherheit zu erhöhen.

Ziel dieser Arbeit ist es festzustellen, ob über eine sogenannte „liquid biopsy“ (flüssige Biopsie; also mithilfe der Entnahme von Körperflüssigkeiten (meist Blut)) zirkulierende DNA/microRNA im Blut und/oder Harn von Patienten mit Fremdorganen zu finden ist, bzw. die Untersuchung von Immunzellen im Blut. Des Weiteren soll festgestellt werden, ob sich die Anzahl im Laufe der Therapie verändert. Dies soll helfen die Diagnostik zu verbessern, um eine effiziente und persönlich angepasste Behandlung und eventuell Früherkennung zu ermöglichen.

## 2. Wie läuft die Studie ab?

Diese klinische Studie wird im Rahmen ihres Routineaufenthaltes an der jeweiligen Klinik durchgeführt, und es werden insgesamt ungefähr 200 Personen daran teilnehmen.



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Eine Reihe von Untersuchungen und Eingriffen werden im Zuge Ihrer Behandlung durchgeführt, gleichgültig, ob Sie nun an dieser Studie teilnehmen oder nicht. Diese werden von Ihrem behandelnden Arzt im Rahmen des üblichen ärztlichen Aufklärungsgespräches mit Ihnen besprochen.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt: Blutentnahme
Wir benötigen für unsere Studie an jedem Zeitpunkt der Routineblutabnahmen und an zusätzlichen Zeitpunkten bei Verdacht auf Abstoßung, bis zu 4x 10 ml (das entspricht circa 4 Esslöffel) für molekulare Analysen (das endgültige Volumen ist abhängig von der während des Projektes evaluierten für die Analysen notwendigen Blutmenge). Unter molekulare Analysen versteht man unter anderem die Gewinnung von DNA/Erbmaterial aus ihren Proben, Durchführung einer PCR, Sequenzierung und Ähnliches.
Die Blutabnahmen werden im Rahmen Ihres studien-unabhängigen Krankenhausaufenthaltes erfolgen. Blut wird Ihnen **im Rahmen der ohnehin vorgesehenen Routineblutabnahmen** entnommen. Dadurch sind für die Studie selbst weder vor noch nach der Diagnose und Behandlung zusätzliche Krankenhausbesuche notwendig. Des weiteren wird während dieser klinischen Studie ihr, im Rahmen der Routinediagnostik abgenommene, Harn im Labor molekularbiologisch untersucht werden. Sollten weitere Harn-Proben notwendig sein wird ihr Arzt sie gesondert darüber informieren.

## 3. Worin liegt der Nutzen einer Teilnahme an der Studie?

Es ist nicht zu erwarten, dass Sie aus Ihrer Teilnahme an dieser Studie gesundheitlichen Nutzen ziehen werden. Auch wenn entsprechende diagnostische oder prognostische Biomarker im Blut identifiziert werden sollten, ist es unwahrscheinlich, dass sich daraus in naher Zukunft therapeutische Konsequenzen ergeben werden. Als mögliche Vorteile sind langfristige Perspektiven zu sehen, da ein besseres Verständnis von molekularen Mechanismen zu neuen Behandlungsansätzen und Möglichkeiten der Therapiesteuerung führen kann, von denen dann andere Personen, welche ein Fremdorgan benötigen, profitieren könnten.

## 4. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

Da die im Rahmen dieser Studie durchgeführten Blutentnahmen im Zuge von für die Routine notwendigen Eingriffen erfolgen, sind keine zusätzlichen Risiken, Beschwerden oder Begleiterscheinungen zu erwarten.

## 5. Wann wird die Studie vorzeitig beendet?



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Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der Studie ausscheiden, ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr Studienarzt wird Sie über alle neuen Erkenntnisse, die in Bezug auf diese Studie bekannt werden, und für Sie wesentlich werden könnten, umgehend informieren.

Es ist aber auch möglich, dass Ihr Studienarzt entscheidet, Ihre Teilnahme an der Studie vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen. Die Gründe hierfür können sein, dass Sie den Erfordernissen der Studie nicht entsprechen, oder dass die gesamte Studie vorzeitig beendet wird. Die Untersuchungen werden solange durchgeführt, bis entsprechende diagnostische oder prognostische Biomarker im Blut identifiziert werden können. Die Studie kann jedoch vorzeitig beendet werden, wenn alle derzeit zur Verfügung stehenden wissenschaftlichen Methoden ausgeschöpft wurden und eine Fortsetzung nicht mehr als sinnvoll erachtet wird.

## 6. In welcher Weise werden die im Rahmen dieser Studie gesammelten Daten verwendet?

Sofern gesetzlich nicht etwas Anderes vorgesehen ist, haben nur die Studienärzte und deren Mitarbeiter Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden („personenbezogene“ Daten). Diese Personen unterliegen der Schweigepflicht. Weiteres können Beauftragte von in- und ausländischen Gesundheitsbehörden oder der zuständigen Ethikkommission Einsicht in diese Daten nehmen, um die Richtigkeit der Aufzeichnungen zu überprüfen. Diese Personen unterliegen einer gesetzlichen Verschwiegenheitspflicht.

Die Weitergabe der Daten im In- und Ausland erfolgt ausschließlich zu statistischen Zwecken in verschlüsselter (nur „indirekt personenbezogener“) oder nicht personenbezogener („anonymisierter“) Form, das heißt, Sie werden nicht namentlich genannt. Auch in etwaigen Veröffentlichungen der Daten dieser Studie werden Sie nicht namentlich genannt.

Die Studienärzte und ihre Mitarbeiter unterliegen im Umgang mit den Daten den Bestimmungen des österreichischen Datenschutzgesetzes und des Gentechnikgesetzes in den jeweils geltenden Fassungen.

Wenn Sie Ihre Einwilligung zurückziehen und damit Ihre Teilnahme vorzeitig beenden, werden keine neuen Daten mehr über Sie erhoben. Aufgrund gesetzlicher Dokumentationspflichten kann jedoch weiterhin für einen gesetzlich festgelegten Zeitraum eine Einsichtnahme in Ihre personenbezogenen Daten zu Prüfzwecken durch autorisierte, zur Verschwiegenheit verpflichtete Personen erfolgen.



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## 7. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser Studie entstehen für Sie keine zusätzlichen Kosten.
Für Ihre Teilnahme an dieser Studie erhalten Sie keine Vergütung.

## 8. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser Studie stehen Ihnen Ihr Studienarzt und seine Mitarbeiter gerne zur Verfügung. Auch Fragen, die Ihre Rechte als Patient und Teilnehmer an dieser Studie betreffen, werden Ihnen gerne beantwortet.

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## 9. Aufbewahrung von Proben

Nach dem Ende der Studie möchten wir die übrigen Proben gerne für weitere Forschungsprojekte auf dem Gebiet der Molekularbiologie verwenden. Für jedes dieser Forschungsprojekte wird zuvor die Zustimmung der Ethikkommission eingeholt. Die Proben werden am Institut für Pathologie der Medizinischen Universität Graz gelagert. Sie können jederzeit verlangen, dass Ihre Proben vernichtet werden. Verantwortlich für die Lagerung und Vernichtung der Proben ist das Institut für Pathologie. Die Proben werden für 30 Jahre gelagert.



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## 10. Einwilligungserklärung

Name des Patienten: ..... Geb.Datum: .....

Ich erkläre mich bereit, an der Studie „Vergleichende molekulare Analysen von zirkulierender DNA/microRNA und Immunzellrezeptor-Repertoire als Biomarker für Gewebsschäden und Transplantatabstoßung“ teilzunehmen.

Ich bin von Herrn/Frau ..... ausführlich und verständlich über mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der Studie, sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser Patientenaufklärung und Einwilligungserklärung, die insgesamt 6 Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Studienarzt verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.
Ich werde den ärztlichen Anordnungen, die für die Durchführung der Studie erforderlich sind, Folge leisten, behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung jederzeit zu beenden, ohne dass mir daraus Nachteile für meine weitere medizinische Betreuung entstehen.
Ich bin zugleich damit einverstanden, dass meine im Rahmen dieser Studie ermittelten Daten aufgezeichnet werden. Um die Richtigkeit der Datenaufzeichnung zu überprüfen, dürfen Beauftragte der zuständigen Behörden beim Studienarzt Einblick in meine personenbezogenen Krankheitsdaten nehmen.
Sollte ich meine Teilnahme an dieser Studie widerrufen oder wird meine Teilnahme an der Studie durch den Studienarzt vorzeitig beendet, so willige ich ein, dass die bis zu diesem Zeitpunkt erhobenen Daten weiterhin verwendet werden dürfen, soweit dies erforderlich ist, um sicherzustellen, dass meine schutzwürdigen Interessen nicht beeinträchtigt werden.
Beim Umgang mit den Daten werden die Bestimmungen des Datenschutzgesetzes und des Gentechnikgesetzes beachtet.

**Für den Fall, dass meine Proben für zukünftige andere Studien interessant wären, bin ich einverstanden, dass meine Proben dort ebenfalls zu Forschungszwecken verwendet werden.**
☐ ja ☐ nein
**Für den Fall, dass ich aus der Studie ausscheide, bin ich einverstanden, dass meine Daten weiterhin verwendet werden:**
☐ ja ☐ nein
**Für den Fall, dass ich aus der Studie ausscheide, bin ich einverstanden, dass meine Proben weiterhin aufbewahrt und analysiert werden, wie in dieser Information beschrieben:** ☐ ja ☐ nein

Eine Kopie dieser Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Studienarzt.

.....
(Datum und Unterschrift des Patienten)

.....
(Datum, Name und Unterschrift des verantwortlichen Arztes)


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# Anhang – Ethikvotum

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## VOTUM

**gültig bis 08.06.2019**

| | |
|---|---|
| **EK-Nummer:** | 30-234 ex 17/18 |
| **Studientitel:** | Comprehensive liquid biopsy in patients after organ transplantation - cfDNA/RNA and immune cell repertoire as marker for tissue damage and organ rejection |
| **Prüfer:** | Dr. Stephan Jahn<br>Medizinische Universität Graz |
| **Sponsor:** | Institut für Pathologie, MUG |
| Ansprechpartner: | Mag. Dr. Karl Kashofer, 8010 Graz, Neue Stiftingtalstrasse 6 |
| **CRO:** | - |
| **Antragsteller:** | Institut für Pathologie |
| Ansprechpartner: | Mag. Dr. Karl Kashofer, 8010 Graz, Neue Stiftingtalstraße 6 |

Die o.a. Studie wurde von der Ethikkommission erstmals im 'expedited Review' am 28.02.2018 behandelt. Die Ethikkommission ist zu folgendem Schluss gekommen:

**Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.**

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: keine

**Zur Beurteilung vorliegende Dokumente:**

| | |
|---|---|
| **Dokumente eingegangen am 16.02.2018, begutachtet im 'expedited Review' am 28.02.2018** | |
| ✓ Cover Letter Anschreiben_v2 2 | 16.02.2018 |
| ✓ Antragsformular ECS | 16.02.2018 |
| Originalprotokoll Studyprotocol_1.1_ cfNA_IMMUNE_TX_2018-01-22 1.1 | 16.02.2018 |
| Informed Consent Form PatientInneninformation cfNA_IMMUNE_TX_Version 1.1 1.1 | 16.02.2018 |
| ✓ CV CV Kashofer 1 | 16.02.2018 |
| ✓ Sonstiges: Antrag um Erlass der Bearbeitungsgebühren 1 | 16.02.2018 |
| ✓ Sonstiges: Zustimmung_Nephro_Transfusion 1 | 16.02.2018 |
| **Dokumente eingegangen am 19.02.2018, begutachtet im 'expedited Review' am 28.02.2018** | |
| ✓ Antragsformular ECS Unterschriftenseiten | 17.02.2018 |
| **Dokumente eingegangen am 28.05.2018, begutachtet im 'expedited Review' am 08.06.2018** | |
| ✓ Originalprotokoll 1.2, undatiert | |
| ✓ Informed Consent Form 1.2 | 11.05.2018 |
| ✓ Sonstiges: Stellungnahme zur Bearbeitungsmitteilung | 28.05.2018 |
| ✓ Sonstiges: Zustimmungserklärung Institut für Transplantationschirurgie | 07.05.2018 |
| ✓ Sonstiges: Stellungnahme der Biobank | 16.02.2018 |
| ✓ Letter of Authorization | 25.05.2018 |

Die Ethikkommission geht – rechtlich unverbindlich – davon aus, dass es sich um ein Projekt mit genetischen Untersuchungen handelt.

Es handelt sich um eine Studie im Rahmen einer Diplomarbeit.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

**zusätzliche Auflagen:** Die Ethikkommission weist darauf hin, dass Proben (nach 2007) ohne gültigen Biobank-IC nur indirekt personenbezogen von der Biobank ausgegeben werden können.

Dieses Votum gilt für ein Jahr ab dem Datum der Ausstellung. Bei längerer Studiendauer ist rechtzeitig vor Ablauf der Gültigkeit des Votums ein Zwischenbericht vorzulegen (Berichtsformular), um eine etwaige Verlängerung zu erlangen.

Graz, 08. Juni 2018

Univ.Prof.DI Dr.Josef Haas
Vorsitzender

Univ.Prof.Dr.Hermann Toplak
Stv. Vorsitzender

**Achtung:** Bitte bei allen das Projekt betreffende Schreiben oder telefonischen Anfragen die EK-Nummer angeben!

# Anhang – Konzeptformular

FB-OSL-04(06)
Konzeptformular für wissenschaftliche Arbeiten

Medizinische Universität Graz

| | |
|---|---|
| **(Arbeits-)Titel**<br>*Das Thema der Diplomarbeit ist einem der im Curriculum festgelegten Prüfungsfächer zu entnehmen.* | Comprehensive liquid biopsy in patients after organ transplant |
| **Untertitel** (optional) | cfDNA/microRNA and immune cell repertoire as markers for tissue damage and organ rejection |
| Konzept erstellt von:<br>Erstellt am:<br>Revisionsdatum bei Änderungen:<br>Version:<br>*(Erste eingereichte Version ist „01“)*<br>Matrikelnummer:<br>Studienkennzahl: | Christian Jurak<br>02-10-2017<br><br><br>01<br>01313688 |
| Betreuer/in:<br>Institut/Klinik:<br>Kontakt:<br>*(Adresse, Telefonnummer, E-Mail)*<br>Zweite/r Betreuer/in:<br>Kontakt:<br>*(Adresse, Telefonnummer, E-Mail)*<br>MitarbeiterInnen: | Kashofer, Karl, Mag. Dr.phil.<br>Institut für Pathologie<br>8036 Graz, Auenbruggerplatz 25/1<br><br>Höfler, Gerald, Univ.-Prof. Dr.med.univ.<br>8036 Graz, Auenbruggerplatz 25/1 |
| **Kernfrage** und Zielsetzung<br>*Wie lautet die Fragestellung?*<br>*Warum ist diese Frage von Bedeutung?*<br>*Welche Ergebnisse sind im Wesentlichen zu erwarten?*<br>*Worin besteht der theoretische Kern der Arbeit?*<br><br>Sind die Forschungsfrage und die mit dem Projekt angestrebte Zielsetzung für Männer und Frauen gleichermaßen bedeutsam? Detaillierte Angaben! | Organversagen bzw. eine Abstoßungsreaktion nach Organtransplantation sind Vorgänge welche prodromal weitestgehend inapparent verlaufen können. Die Analyse von Nukleinsäuren aus dem Blut (Liquid Biopsy), sowie die Diagnostik der T-Zell Klonalität haben in rezenten Studien die Möglichkeit ihrer Verwendung als Biomarker gezeigt.<br>Durch ihre minimale Invasivität (Blutabnahme) hat die „Liquid Biopsy“ Vorteile gegenüber etablierten Methoden und könnte auch in Hinblick auf Sensitivität und Spezifität Vorzüge zeigen.<br>Ziel ist es durch eine ausführliche Literaturrecherche und experimentelles Arbeiten, Labormethoden zu etablieren um bei Patienten mit Organtransplantation eine Liquid Biopsy auf microRNA, cfDNA und T-Zell Ebene durchführen zu können. Nach dem Aufbau der Methoden sollte ein „Proof of Concept“ mit einer kleinen Patientenkohorte stattfinden.<br>Das Geschlecht ist für die angestrebte Patientenkohorte kein Ein/Ausschlusskriterium und resultierend daraus sind auch die Ergebnisse für beide Geschlechter gleich relevant. |
| **Kurzbeschreibung** (max. 20 Zeilen)<br>*Worin besteht der Neuigkeitswert?* | Die Verwendung von „Liquid Biopsy“ für das Monitoring von Transplant-Patienten steckt noch in den Kinderschuhen. Studien zeigen jedoch Vorteile gegenüber etablierten Methoden.<br>Im Rahmen dieser Arbeit sollen die oben genannten Methoden für die molekulare Diagnostik der Pathologie Graz etabliert werden und möglicherweise einen Beitrag zum besseren Monitoring für Transplant-Patienten leisten. |

| | |
|---|---|
| **Methodenwahl**<br>*Welche Methoden stehen zur Beantwortung der Frage zur Verfügung? Wieso wählen Sie genau diese Methode?* | Methoden der molekularen Diagnostik auf Nukleinsäure-Ebene werden für die Diplomarbeit verwendet: RNA-Seq , PCR, T-Cell Profiling et ceterae. |
| **Ethikkommissionsvotum**<br>*Ist ein Votum der Ethikkommission erforderlich?*<br>*Siehe Informationsblatt „Genehmigung Ethikkommission“* | X Erforderlich<br>☐ Nicht erforderlich<br>☐ Bereits vorhanden |
| **Datenerhebung** (falls zutreffend)<br>*Werden aufgrund der oben genannten Methodenwahl medizinische Daten benötigt? Wenn ja, welche?*<br>*Mit welcher Fallzahl ist zu rechnen? Wie wurde die Fallzahl ermittelt?*<br>*Wie ist das PatientInnenkollektiv zu beschreiben (Mindest-/Höchstalter, Geschlechtsverteilung, Begleiterkrankungen, etc.)?*<br><br>*Bitte beachten Sie, dass eine Weitergabe der Daten an projektfremde Personen gemäß Datenschutzgesetz nicht zulässig ist. Das Bekanntwerden von PatientInnendaten ist durch Pseudonymisierung (Codierung mit fortlaufender Nummer) und ggf. Zugriffsbeschränkungen zu verhindern.* | Das Diplomarbeitsprojekt dient als Vorlage für eine weiterführende Studie welche sich auf 2 Jahre erstreckt und mit den hier etablierten Methoden eine größere Kohorte bedient.<br><br>Die benötigten medizinischen Daten belaufen sich auf Blutproben und krankheitsrelevanten Daten der Patienten.<br><br>Das Kollektiv, 18-99 Jahre, sollte eine ausgeglichene Geschlechterverteilung aufweisen, jedoch ist aufgrund des Studiendesigns der übergreifenden prospektiven Studie eine genaue Verteilung nicht vorhersehbar. Einschlusskriterium sind Patienten mit Organtransplantation. |
| **Datenauswertung**<br>*Welche Hauptzielgröße wird analysiert (z.B. Alter bei Diagnosestellung/Alter bei Operation/Diagnose, etc.)? Wie wird die Hauptzielgröße analysiert?*<br>*Welche Nebenzielparameter sollen betrachtet werden? Mit welchen Methoden erfolgt die Auswertung?* | Der Kern der Arbeit ist die Etablierung der Labormethoden.<br><br>Die Zielgrößen belaufen sich auf cfDNA-Menge (quantitiv) im Blut, bzw. microRNA-Profiling und T-Zell Klonalität bei Patienten nach Organtransplantation. |
| **Zeitplan** (grob strukturiert)<br>*Wann wird mit der Arbeit begonnen? Wann wird ein Antrag bei der Ethikkommission gestellt, sofern ein Votum erforderlich ist? Welche Meilensteine wurden zwischen dem/der Studierenden und den BetreuerInnen vereinbart? Wann ist voraussichtlich mit der Beendigung der Arbeit zu rechnen?*<br>*Welche formalen Schritte sind für die Umsetzung der Diplomarbeit notwendig?* | Der Beginn ist mit Anfang Wintersemester 2017/2018 angesetzt und soll sich auf die Dauer des gesamten Studienjahres 2017/2018 erstrecken.<br><br>Antrag bei der Ethikkommission wird mit KW 41 (2017) gestellt<br><br>Meilensteine sind die Etablierung der einzelnen Labor-Methoden und das darauffolgende Proof-of-Concept mit einer kleinen Patienten-Kohorte.<br><br>Die formalen Schritte belaufen sich auf das Einholen eines Votums der Ethikkommission, das Erstellen des Studienprotokolls, informierte Einwilligung und des Konzeptformulars. |
| **Referenzen**<br>Welche Literatur ist relevant? Gibt es Vergleichsstudien? | [1] Gielis, E. M. et al. Cell-Free DNA: An Upcoming Biomarker in Transplantation. American Journal of Transplantation 15, 2541–2551 (2015).<br><br>[2] Schütz, E. et al. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: A prospective, observational, multicenter cohort study. PLoS Med. 14, e1002286 (2017).<br><br>[3] Hamdorf, M., Kawakita, S. & Everly, M. The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection. J Immunol Res 2017, 4072364 (2017).<br><br>[4] Celen, E., Ertosun, M. G., Kocak, H., Dinckan, A. & Yoldas, B. Expression Profile of MicroRNA Biogenesis Components in Renal Transplant Patients. Transplant. Proc. 49, 472–476 (2017). |

| | |
|---|---|
| | [5] Zhou, M. et al. Circulating Organ-Specific MicroRNAs Serve as Biomarkers in Organ-Specific Diseases: Implications for Organ Allo- and Xeno-Transplantation. Int J Mol Sci 17, (2016).<br>[6] Alachkar, H. et al. Quantitative characterization of T-cell repertoire and biomarkers in kidney transplant rejection. BMC Nephrol 17, 181 (2016). |
| **Benötigte Ressourcen**<br>*Werden Geld- oder Sachmittel von Einrichtungen der Med Uni Graz benötigt?*<br>Die Vergabe ist nur zulässig, wenn die Leiterin/der Leiter dieser Einrichtung über die beabsichtigte Vergabe informiert wurde und diese nicht binnen eines Monats untersagt hat. | JA<br>Das Institut für Pathologie stellt im Rahmen der wissenschaftlichen Studie Räumlichkeiten und Materialen zur Verfügung. |

# Anhang – Biobank Graz Projektanfrage

| BioBank GRAZ | **O-FIS Qualitätsmanagementsystem**<br>Formblatt<br>**Projektanfrage** | FB310<br>Seite1 von 2 |
|---|---|---|

**An der Biobank Graz kann ausschließlich um biologische Proben volljähriger SpenderInnen angefragt werden.**

**Grüne Felder werden von der Biobank Graz ausgefüllt.**

| ☒ **Neue Anfrage** | ☐ **Ergänzung / Erweiterung zu Projekt Nr.** |
|---|---|
| **Datum der Anfrage:** | 16.02.2018 |
| **Projektnummer:** | 1004_18 |
| **Titel des Forschungsprojekts:** | Comprehensive liquid biopsy in patients after organ transplantation – cfDNA/RNA and immune cell repertoire as markers for tissue damage and organ rejection |
| **ProjektleiterIn:** | Mag.Dr. Karl Kashofer |
| **AntragstellerIn:**<br>(nur auszufüllen wenn nicht ident mit ProjektleiterIn) | |
| **Klin. Abteilung / Institution:** | Institut für Pathologie |
| **Kontaktdaten:**<br>(Adresse, Telefonnummer, E-Mail) | Institut für Pathologie, Neue Stiftingtalstraße 6, A-8010 Graz<br>+43-316-38571752<br>karl.kashofer@medunigraz.at |

1. **Inhalt der Anfrage (z.B.: Fragestellung, Anzahl und Art der Proben, Selektionskriterien, bei internen Projekten Liste der Histonummern beilegen)**

In Ergänzung zur Methodenetablierung der Projektnummer 1002_18_Niere und 1003_18_Leber werden für diese Proben auch für ein Forschungsprojekt - im Rahmen einer Diplomarbeit - verwendet. Wir benötigen 5x Proben von gesundem Nierengewebe (Kryo) und 5x Proben von gesunden Lebergewebe (Kryo) um daraus microRNA zu isolieren.

2. **Art der Anfrage**

| ☒ **Forschungsprojekt** | ☐ **Methodenetablierung** | ☐ **Universitäre Lehre an der Med Uni Graz oder Vortragsvor-bereitung** |
|---|---|---|
| Biobank Projektantrag FB311 und Beilagen entsprechend Ethikkommission erforderlich*. | Ausgabe der Proben von max. 5 PatientInnen im Rahmen einer Pilotstudie ohne Pub-likation. | Nur Ausgabe von Original-schnitten, keine Ausgabe von Blöcken für die Lehre. |
| **Anmerkung:** | | |

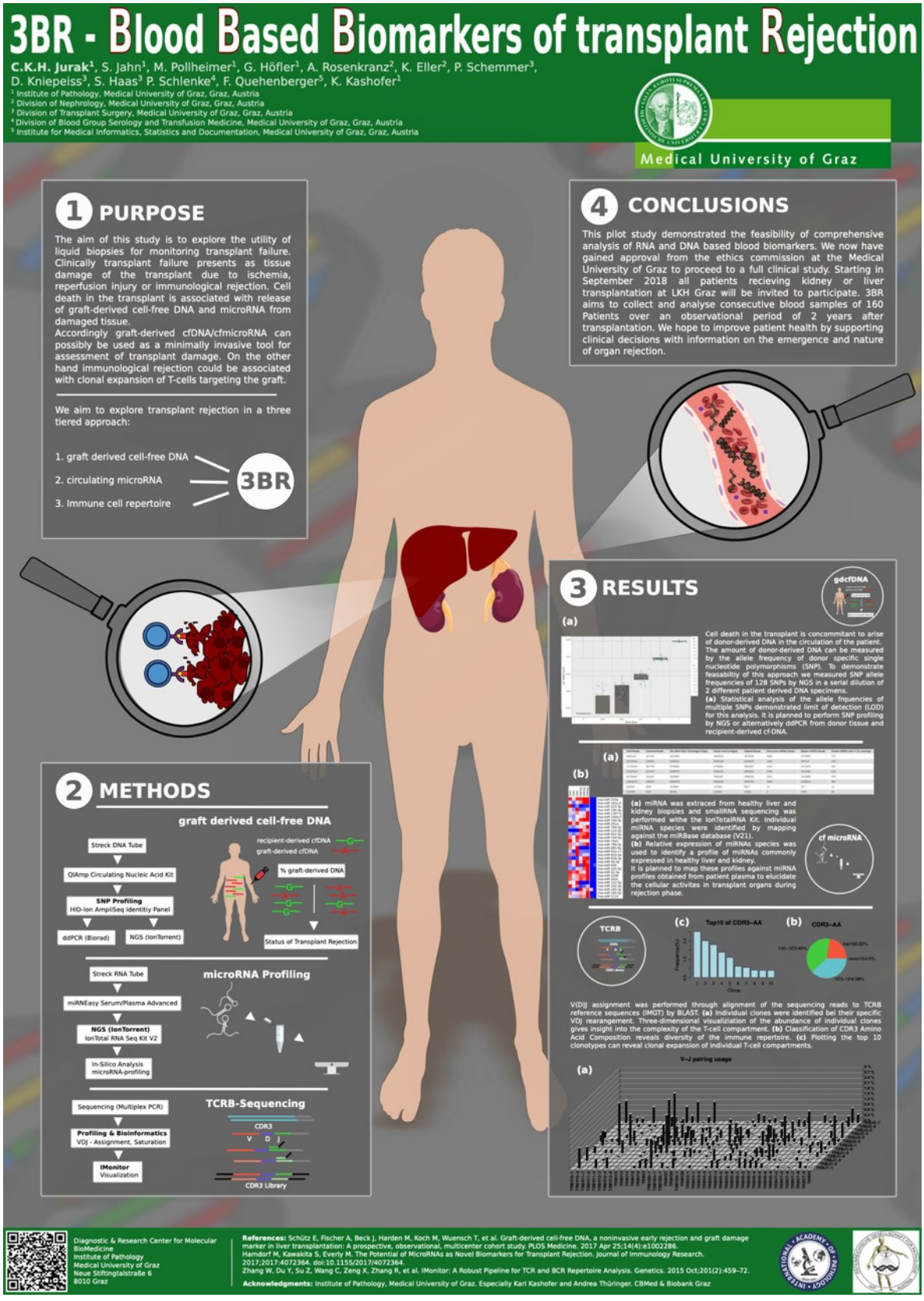
* Ethikantrag und Votum, Studienprotokoll und anderweitig der Ethikkommission vorgelegte Dokumente (z.B. studienspezifischer Informed Consent)

3. **Rückmeldung / Stellungnahme der Biobank Graz**

Die Biobank Graz kann die in der Routine gesammelten Proben von Leber und Nieren Normalgewebe (soweit vorhanden) für dieses Projekt zur Verfügung stellen.
Da für diese Studie auch Cryogewebe, das nach dem Jahr 2007 archiviert wurde, ausgewählt wird, kann nicht ausgeschlossen werden, dass Proben nach dem Jahr 2007 ohne Biobank Informed Consent angefragt werden.

Die Proben nach 2007, für die kein gültiger Biobank Informed Consent vorhanden ist, können an die Projektgruppe weitergegeben werden (DSG §46, Abs. 1), sofern die Ethikkommission durch Ausstellung eines positiven Votums diesem Vorgang zustimmt.
Die Biobank Graz weist ausdrücklich darauf hin, dass die Verantwortung für den pseudonymisierten Umgang und die anonymisierte Weitergabe der für diese Studie angefragten Proben sowie der zugehörigen Daten ausschließlich beim Projektleiter liegt.
Die Probenausgabe durch die Biobank Graz erfolgt vorbehaltlich der Stellungnahme der Biobank-Beauftragten und den Genehmigungen des Approval Committees sowie der lokalen Ethikkommission.

# Anhang – Herbsttagung 2018 der ÖGPath/IA Austria

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