Tutor/s

Dr. Rafael Franco Fernandez
Departament de Bioquímica i Biologia Molecular

Dr. Mercè Brunet Serra
Farmacologia i Toxicologia. SBGM. Hospital Clinic

Grau de Química

Treball Final de Grau

miRNA-155-5p, miRNA-181a-5p, and miRNA-122-5p as prognostic biomarkers of clinical graft evolution in liver transplant patients.
Els miRNA-155-5p, miRNA-181a-5p i miRNA-122-5p com a biomarcadors pronòstics de l'evolució clínica de l'empelt en pacients trasplantats hepàtics.

Eva Marone Millà

January 2022

Universitat de Barcelona

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

En algún lugar, algo increíble está esperando a ser conocido

Carl Sagan

Primer de tot, m'agradaria agrair a la Dra Brunet per la seva dedicació i paciència durant la meva estada. En aquest mesos he après molt gràcies a les conferències i sessions de teoria i pràctica que m'han impartit en el laboratori de l'Hospital Clínic. També vull agrair a la Dra Millán per tot el temps dedicat en ensenyar-me la metodologia i funcionament del laboratori. He estat molt a gust gràcies a la seva acollida i a la dels tècnics del laboratori. Al Dr Franco per seguir el desenvolupament treball i ajudar-me en la redacció. I sobretot agrair a la meva família i amics per ser-hi sempre, encara que sigui moralment.

REPORT

CONTENTS

1. SUMMARY

Liver transplanted patients receive strong immunosuppressive drugs with the aim of preventing allograft rejection. Although nowadays new drugs and surgical techniques and technologies are being used, the diagnose of allograft rejection is still a concern. This is due to the conventional biomarkers that are used in order to diagnose allograft rejection. Their values are often abnormal, or they are not able to discriminate this disease from others, and thus it is needed to perform a biopsy which is related with complications. It is though needed new sensitive and specific biomarkers for screening and monitoring liver transplanted patients. miRNAs are a subtype of noncoding RNAs which have been studied the past decades and appear to be biomarkers related with several diseases. In this study, we are going to evaluate different miRNAs (miRNA-155-5p, miRNA-181a-5p and miRNA-122-5p), in order to predict allograft rejection. Their analysis will be performed via quantitative real-time polymerase chain reaction, and we will obtain their expression in plasma. Rejector patients will be compared to non-rejector patients through statistical treatment of the miRNAs expression.

Keywords: miRNAs, biomarkers, graft rejection, liver transplantation, personalized medicine

2. RESUM

Els pacients trasplantats de fetge reben fàrmacs immunosupressors potents amb l'objectiu de prevenir el rebuig de l'al·loempelt. Encara que avui dia s'estan utilitzant nous fàrmacs i noves tècniques i tecnologies quirúrgiques, el diagnòstic de rebuig d'al·loempelt segueix sent una preocupació. Això es deu als biomarcadors convencionals que s'utilitzen per diagnosticar el rebuig de l'al·loempelt. Sovint els seus valors són anormals, o no són capaços de discriminar aquesta malaltia de les altres, i per això cal fer una biòpsia la qual està relacionada amb complicacions. Tanmateix, es necessiten nous biomarcadors sensibles i específics pel seguiment i monitoratge pacients trasplantats de fetge. Els miRNA són un subtipus d'ARN no codificant els quals han estat estudiats durant les últimes dècades i semblen ser biomarcadors relacionats amb diverses malalties. En aquest estudi, avaluarem diferents miRNAs (miRNA-155-5p, miRNA-181a-5p i miRNA-122-5p), per tal de predir el rebuig de l'al·lograft. La seva anàlisi es realitzarà mitjançant una reacció quantitativa en cadena de la polimerasa en temps real, i obtindrem la seva expressió en plasma. Els pacients que rebutgin l'empelt es compararan amb els pacients que no hagin rebutjat mitjançant el tractament estadístic de l'expressió de miRNAs.

Paraules clau: miRNAs, biomarcadors, rebuig de l'empelt, transplantament hepàtic, medicina personalitzada

3. INTRODUCTION

Over the past decades, liver transplanted patients have been a clinical concern due to the low survival rates. Nevertheless, nowadays these rates have been improved, especially the first years after the transplantation, due to precise surgical techniques and technologies, more effective immunosuppressive (IS) drugs and a better and early diagnosis and management after liver transplantation (LT).[1] On the other hand, pharmacological treatments and, especially immunosuppression have a notable impact in graft function and outcome. Unfortunately, IS agents such as tacrolimus, cyclosporine, everolimus and sirolimus are associated with severe adverse events that may negatively influence graft and patient clinical outcome, early or long term after transplantation.

The first few weeks after transplantation, when the risk of allograft rejection (AR) is high, patients receive strong immunosuppression treatment with the aim to prevent this clinical event. AR is an important cause of morbidity, allograft lose, and it can also lead to mortality.[2] Although IS therapies are effective in preventing patients from AR, there is also a requirement to prevent their side effects.[3–5]

Rejection may be acute, chronic, or subclinical, the last one due to inflammatory lesions in the allograft (SCR). In addition, the most usual rejection in liver transplant recipients is acute T-cell mediated rejection (TCMAR) that occurs in 21–27% of the patients.[6] The activated T-cells, differentiate into cells which can migrate and destroy the allograft.[7] In the context of this population, SCR that may affect about 15% of transplanted patients, is also of interest because it reflects the maintained alloreactivity against the implanted graft, although a full rejection is not occurring, it negatively impacts on graft function. Regarding antibody mediated rejection (ABMR), few liver transplant patients may suffer this kind of rejection although it is of high incidence in kidney transplant recipients.[8] Chronic rejection may occur in 2-3% of patients several months or years after transplantation.[6]

IS drugs have a high inter and intra patient pharmacokinetic (PK) and pharmacodynamic (PD) variability. So, to minimize drug-related side effects and improve efficacy, therapeutic drug

monitoring (TDM) is a requirement for personal dose adjustment with the aim to achieve blood target concentrations. TDM and personalized therapy are needed due to the different variability's inter and intra patients and to improve efficacy. TDM including screening patient's response to IS drugs may be studied by PK, which contemplates the time course of drug absorption, drug blood concentration and half-life, drug metabolism, and its excretion; and PD, based on the analysis of biomarkers that may reflect the immunomodulatory effects of the drugs in each treated patient. Treatment monitoring also includes correlation with the modifiable risk factors in patients such as age, sex, lifestyle, and adherence to the treatment. [9–11]

A holistic point of view is necessary for the selection of the most effective and safe IS treatment that should be based on pharmacokinetic, pharmacogenetic and pharmacodynamic variables.

This TFG project focuses on evaluating the correlation between microRNA (miRNA) biomarkers and the risk of TCMAR.

3.1. BIOMARKERS IN LIVER TRANSPLANT

In the past years, several biomarkers have been evaluated to prevent and diagnose AR and to follow the IS treatment in liver transplanted patients. Nevertheless, a single biomarker may not be able to discriminate rejection from other pathologies due its clinical complexity, therefore, after transplantation. Non-invasive biomarkers such as biomarkers in the saliva, peripheral blood, urine, or other body fluids are needed because liver biopsy is an invasive procedure associated with severe complications. These biomarkers should be also available, precise, cost effective, and robust and should not require a too complex technique.[12] It is also very important to present high sensitivity to ensure reliability in the identification of patients suffering from AR; and high specificity, to discriminate AR from other diseases such as liver dysfunction and cytomegalovirus or hepatitis C virus infections. Liver transplanted patients are monitored by standard blood tests using current biochemical markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), alkaline phosphatase, creatinine and bilirubin.[2, 13] However, these serum biomarkers have shown a low specificity and sensitivity for AR.[14] Moreover, those tests often show inconclusive values thus leading to the requirement of allograft biopsy.[15] Hence, it is needed other more specific and sensible biomarkers to diagnose and predict AR. In the last reports, different biomarkers were tested to monitor liver transplanted patients. Pro-inflammatory and immunoregulatory cytokines

such as (IL)-2 and (INF)- γ, measured in T-cell by flow cytometry, seem to be suitable biomarker candidates as they can regulate the immune response and their production and secretion can be modify by IS drugs.[16] Although these cytokines seem to be useful to diagnose rejection, they cannot usually discriminate rejection from infections and might also be modified by specific bacterial and viral infections.[12] Chemokines (CXCs) are also involved in the immune response after transplantation.[18] It is reported that CXCL-9 and CXCL-10, are increased in rejecting renal allografts.[17] *Raschzok* et al, reported that CXCL9 was significantly higher in the pretransplant and at the first day after LT[19], and *Friedman* et al. showed that CCL-2, CXCL-9 and CXCL-10 were related with allograft dysfunction.[20] In addition, *Zhang* et al. showed that in patients with AR, the receptors CXCR3−CCR6−CXCR5+CD4+ T cells may promote AR after LT, and that the receptors CXCR3−CCR6+CXCR5+CD4+ T cells may suppress it. However, there is not enough information on the potential of these chemokines and their specific receptor as biomarkers in LT, and their analyses by flow cytometry require too complex techniques to be implemented in a clinical context.[21]

Regarding pharmacodynamic biomarkers, it is suggested to measure the levels of the nuclear factor of activated t-cell regulated transcription factor (NFAT), IL-2, IFN-γ and granulocyte macrophage colony-stimulating factor. Determination of residual NFAT-regulated gene expression can diagnose infections, malignancy, acute rejection, and cardiovascular risk.[12]

In conclusion, it is needed new improved biomarkers, non-invasive and not too complex to quantify, to enhance personalized monitoring. In this study, we've analyzed miRNAs as biomarkers to evaluate rejection in LT.

3.1.1. microRNAs

Noncoding RNAs (ncRNAs) are RNA molecules that are not translated into proteins. Depending on their size, there are different types of ncRNAs. Long ncRNAs contain more than 200 nucleotides, and short ncRNAs, contain less than 200 nucleotides. miRNAs are a subtype of short ncRNAs that contain around 21-24 nucleotides.[22]

In 1993, the first miRNA, lin-4, was discovered in the nematode Caenorhabditis elegans by *Ambros* et al. [23] Its hairpin structure is shown beyond (figure 1) as a computer prediction. [24]

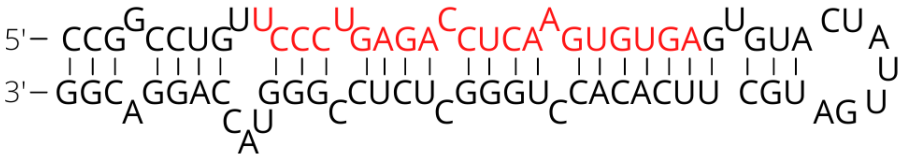

Figure 1. lin-4 computer-predicted structure. Red bases are mature miRNA sequences.

MicroRNAs, miRNAs or miRs have been studied in the past years and seem to be promising next-generation biomarkers in clinical practice.

These molecules are posttranscriptional regulators and can inhibit posttranscriptional gene expression by inducing mRNA degradation and translational repression.[25] miRNAs bind to a specific sequence of target messenger RNA (mRNA) resulting the inhibition of *de novo* proteins and suppressing translation by the degradation of target mRNA. Depending on the degree of complementary between miRNAs and target mRNA, mRNA will be degraded or repressed. [26] A perfect sequence complementarity leads to degradation while in translational repression the sequence complementary is partial.[27-32] The interaction between miRNAs and their target depends on many factors, for instance the subcellular location of miRNAs, the amount of miRNAs and target mRNAs, and the affinity of miRNA and mRNA complementary.[26] It is thought that a single miRNA can target different of mRNAs and that a single mRNA may be targeted by different miRNAs.[22] miRNAs regulate different cellular actions including cell growth, differentiation, development, and apoptosis[30] and they also participate in the cellular mechanisms of inflammatory response. [31] Their expression is specifically altered in pathological conditions and diseases[33] and evaluation of miRNA expression could be used to discriminate alloimmune injuries, such as AR, from regeneration events.

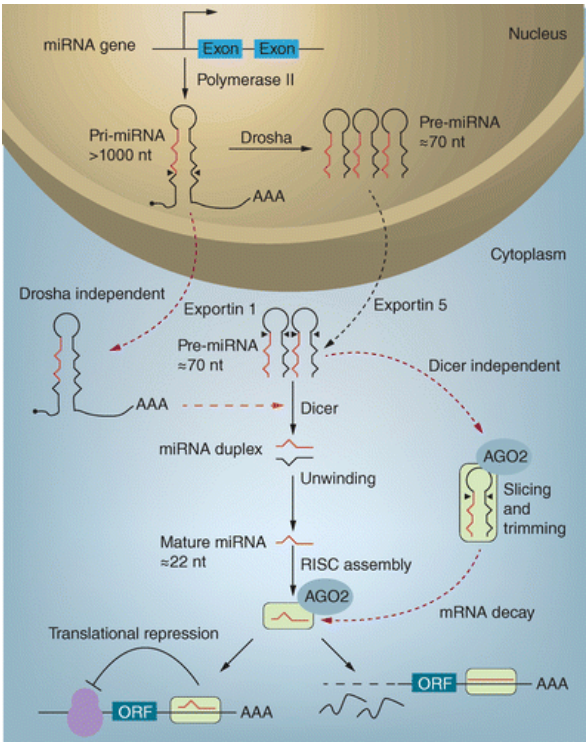

Figure 2. miRNA biogenesis and mechanism of mRNA degradation or translation inhibition. (Image extracted from Bernardo, B. C. ref 32)

miRNA-coding DNA sequences have different locations in the genome. miRNAs are transcribed from DNA sequences, mainly from introns, which are sequences that do not code for proteins. Primary miRNAs are processed further into precursor miRNAs (pre-miRNA), and finally to mature miRNAs. There exist different pathways of miRNA biogenesis and function is shown in figure 2. The canonical pathway which is the most common is shown in black arrows. One strand of the mature miRNA associates with argonaute2 protein (AGO2) forming the miRNA-induced silencing complex (RISC). The full complementary of RISC with target mRNA, produces its degradation and the mismatch of sequences causes translational inhibition.[32]

The main action of miRNA is to inhibit gene expression, as mentioned previously. Nevertheless, some studies have reported that there exist miRNAs, for instance, let-7, that regulate gene transcription.[22]

Circulant miRNAs are secreted in all biological fluids such as urine, serum, plasma, and saliva, and are transported to target cells through vesicles, especially exosomes, or by binding to proteins, e.g., argonautes. Blood circulant miRNAs may have a role as signaling molecules to mediate cell-cell communications.[26] They are highly stable in fresh, frozen and paraffined samples and they can be analyzed by numerous techniques: real time quantitative polymerase chain reaction (qPCR), microarray, multiplex bead-based flow cytometry, next-generation sequencing and in-situ hybridization. When the aim is to detect individual miRNAs and the sample amount is a limiting step, qPCR is commonly used. If several samples are screened in order to find new or altered miRNAs, the use of microarrays is recommended. On the other hand, if the intention is to find different isoforms or silent miRNAs and discover novel miRNAs, new generation sequencing is performed. Actually, qPCR is the most used technique for circulating miRNAs quantification owing to its sensitivity.[34]

Although mRNA degrades in urine, miRNAs remain highly stable due to their exosome protection from ribonuclease activity, which hydrolyses mRNA. Urinary miRNAs may be related to inflammation processes, but their potential diagnostic capacity remains uncertain. The transforming growth factor beta (TGF-ß) signaling pathway affects many cellular processes such as cell growth, differentiation, or migration. TFG-ß signaling pathway is the main pathway for organ rejection, and miRNAs may play a role in this immunoregulation.[26] In current studies, different miRNAs have been taken in consideration in order to diagnose or discriminate diseases or pathologies, depending on the relation between the miRNAs and the target genes or their response. miR155-5p mediates the inflammatory response and regulates IFN-γ production in T-cells and non-killer cells. Also, miR-122-5p is the most abundant miRNA in liver, being 70% of total miRNA in the liver. Hence, a significant increase of this miRNA may be related with damage, toxicity, or viral infection. miRNA-181a-5p can regulate T-cell response in the process of allorecognition, which is crucial in AR. [35] Their computer-predicted structures are shown beyond in figures 3, 4 and 5.

Figure 3. Computer-predicted structure of Premir-122. Red sequences are mature miRNA sequences.

Figure 4. Computer-predicted structure of Premir-155. Red sequences are mature miRNA sequences.

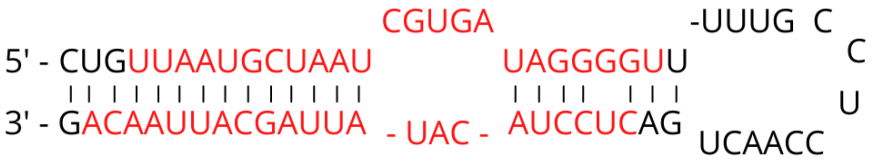

Figure 5. Computer-predicted structure of Premir-181. Red sequences are mature miRNA sequences.

Farid et al. reported that, at the beginning of rejection, the miR-122 reaches a greater relative increase in serum than that of the classical aminotransferase marker thus making possible earlier diagnosis and faster intervention.[36] A study by *Ruiz* et al. showed a significantly higher plasma expression of miRNA 155-5p, 122-5p and 181a-5p in patients with TCMAR in comparison with rejection-free patients. Although the mechanisms of the alteration in the level of miRNAs in TCMAR or AR after LT remains unclear, miRNAs are capable of discriminate TCMAR from other pathologies.[37] A study by *Millán* et al. reported that in pre-transplantation, miR-155-5p expression was significantly higher in TCMAR patients and, interestingly, in SCR patients, the miR-181a-5p expression was also significantly higher compared with rejection-free

patients. The study also showed that TCMAR and SCR patients displayed earlier miR-181a-5p, miR-155-5p, and miR-122-5p increases than transaminases. In summary, pre-transplantation the analysis of plasmatic miR-155-5p expression may be useful for identifying low-risk immunologic patients and provides guide to select IS, and post-transplantation the monitoring of miR-181a-5p and miR-155-5p may be useful to prevent TCMAR or SCR.[6] Owing to the small sample size and the case-control study design, multicenter trials are needed to provide clearer clinical evidence.

3.2. IMMUNOSUPPRESSIVE THERAPY

As mentioned previously, all the transplanted patients receive a chronic treatment, based on IS drugs. However, it is very important in transplanted patients to personalize the IS therapy because high concentration can lead to toxicity, and low concentration may not be enough from preventing AR.

Post LT IS therapy consists of two phases: induction and maintenance. IS induction occurring in the first months after LT and it is an intense therapy which protects the allograft against acute rejection. After IS induction, its maintenance treatment is prescribed, which is a life-long treatment for the patient in order to preserve the allograft.[37]

Most IS drugs are lipophilic molecules with high molecular weight, and they are products of the metabolism of fungi.[38]

Different types of IS drugs are being prescribed to patients. For instance, calcineurin inhibitors (CNI) is a group of IS drugs which include cyclosporine and tacrolimus (Tac). High values of CNI can lead to a major risk of nephrotoxicity, which leads to the need to institute renal replacement therapy and carries increase in mortality risk.[37] The mammalian target of rapamycin inhibitors is another group of IS drugs which include drugs such as sirolimus and everolimus. These drugs regulate cytokine production to inactive T-cell thus preventing AR. Another IS drugs are precursors of mycophenolic acid, a purine synthesis inhibitor, and azathioprine, a pyrimidine synthesis inhibitor. There is also available antibody therapy when CNI inhibitors cannot be used.[37,38] On the other hand, Tac is the main IS drug used in solid organ transplantation.[37]

Mostly IS drugs are metabolized by the CYP3A4 system. Tac is metabolized by CYP3A4 and CYP3A5 in liver and intestine, with less contribution of CYP3A7 system. The *CYP3A5*

genotype influences Tac dose requirement in patients. A patient with *CYP3A5*3* allele, is associated with decreased enzyme expression and is, therefore, a poor metabolizer; in contrast individuals presenting *CYP3A*1/*3* or *CYP3A5*1/*1* alleles, are intermediate and fast metabolizers, respectively. The poor metabolizers will need conventional or low doses of Tac while fast metabolizers will need higher doses to achieve efficacy, hence genotyping is recommended to recalculate personal dose requirement. [12,39,40]

4. OBJECTIVES

In line with the tasks in a FIS-granted Project (FIS PI19/00378), the main goal of this TFG project is to analyze different miRNAs (miRNA-155-5p, miRNA-181a-5p, and miRNA-122-5p) expression in plasma samples as prognostic biomarkers of clinical evolution of the allograft in liver transplanted patients.

The hypothesis is that these miRNAs will be able to identify among liver transplanted patients those at high risk of AR.

This study will be carried via experimental techniques, and statistical treatment of the results. In the studied population, the miRNAs from patients with acute rejection will be compared to those patients free of rejection (control group).

5. EXPERIMENTAL SECTION

5.1. POPULATION OF STUDY

25 Patients were included in this study, as part of a competitive project funded by the Instituto de Salud Carlos III (project FIS PI19/00378), which is currently being developed in the Laboratory of Pharmacology and Toxicology of the Biochemistry and Molecular Genetics Service of Hospital Clinic of Barcelona. It was approved by the Ethics Committees of the center and all patients were previously informed and provided written consent (Reg. HCB/2019/0258).

Adult *de novo* liver-transplanted patients of the Hepatic Transplant Unit of Hospital Clinic, are monitored from pre-transplantation to one year after transplantation, and the plasma expression of a panel of miRNAs (miR-155-5p, miR-122-5p and miRNA-185a-5p) is determined.

The main inclusion criteria are patients over 18 years old, transplanted de novo, HIV negative, which agree with the terms of the study and sign the informed consent.

The IS treatment that patients receive is adjusted following the usual guidelines of the liver transplant unit of the hospital clinic, being the most common the combination of tacrolimus, mycophenolate mofetil and prednisone.

In case of suspicion of AR, the allograft biopsy is performed according to the usual procedure, and this rejection is classified according to the Banff classification. In moderate-severe cases of TCMAR, steroid bolus is used. In SCR cases, rejection or maintained alloreactivity in biopsy is seen although biochemistry or clinics abnormalities are not manifested.

This study includes 25 patients, which 13 are follow-up patients, and 12 are *de novo* transplanted patients.

5.2. REAGENTS

All reagents were purchased from commercial sources. Different kits from QIAGEN® were used in sample analysis: miRNeasy Serum/Plasma Advanced Kit (cat. No. 217204) for purification of total miRNA from plasma, miRCURY LNA® RT Kit (cat. No. 339340) for reverse transcription of miRNA, and miRCURY LNA SYBR® Green PCR Kit (cat. Nos. 339345, 339346 and 339347).

5.3. SAMPLING

In order to analyze miRNAs in plasma, blood samples from liver transplanted patients included in the study are collected into EDTA-K3 tubes (3ml). The samples are obtained preceding the IS administration (pre-dose), and before any change in IS therapy was done. The frequency for sample collection are 1 week, 15 days, 1 month, 2 months, 3 months and 6 months after LT, and at any time when patients have a high probability of suffering AR. After centrifugation (within 2h) at 3000 x g for 10 min to remove cells, plasma was collected and stored in RNase-free tubes at -70ºC, until their analysis.

5.4. PURIFICATION OF MIRNAS FROM PLASMA

The general procedure for miRNAs analyses is described in figure 6 and includes 3 steps. First, after obtaining blood sample from patients, miRNAs from plasma are purified. Next, reverse transcription reaction (RT) is performed in order to obtain cDNA. After that, qPCR is performed to amplify the cDNA sequence of interest, in this case, those which belongs to each miRNA. C_t values which correspond to the level of expression of each miRNA are obtained by Roche LC Software during qPCR.

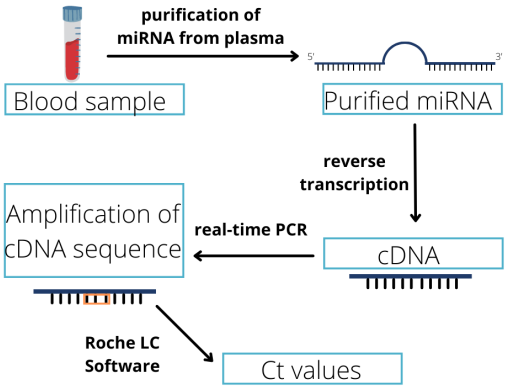

Figure 6. General procedure for miRNAs analyses.

Blood plasma is a type of sample which needs RNA purification procedures, and usually, the concentration of RNA in the samples cannot be precisely determined and it is very low in plasma. Thus, it is needed an RNA carrier (1 µg RNA-MS2 per sample) in the purification procedure to ensure RNA isolation and to improve its yield.

The following steps are performed according to the manufacturer's protocol of miRNeasy Serum/Plasma Advanced Kit, where total RNA was purified from 200 µl of patient plasma.

- First of all, plasma cells were lysed with 60 µl of the lysis solution, which contains 58,7 µl of Buffer RPL and 1,3 µl of MS2, and mixed by vortexing for more than 5 s. Then, it is left at room temperature for 3 min.

- Next, proteins were precipitated by adding 20 µl of RPP buffer and mixed vigorously for more than 20 s. It is then centrifuged at 12,000 x g for 3 min at room temperature.

- The supernatant is transferred to a new reaction tube and 1 volume of isopropanol (of 230 µl approximately) is then added. It is mixed by vortexing, then transferred to an RNeasy UCP MinElute column and centrifuged for 15 s at more than 8000 x g. The flow-through is discarded.

- At this point, different reagents are added to clean the column. First, 700 µl of Buffer RWT are added to the column and centrifuged for 15 s at more than 8000 x g. The flow-through is discarded. After that, 500 µl of Buffer RPE are added to the column and centrifuged for 15 s at more than 8000 x g. The flow-through is discarded. Then, 500 µl of 80% ethanol are added to the column and centrifuged for 15 s at more than 8000 x g. The flow-through and the collection tube are discarded.

- The column is placed into a new 2 ml collection tube. The lid is opened and centrifuged at 5 min in order to dry the membrane. The flow-through and the collection tube are discarded.

- The column is placed in a new 1,5 ml collection tube and 20 µl of RNase-free water are added to the center of the column membrane and it is incubated 1 min. Then, the lid is closed and centrifuged for 1 min at full speed to elute the RNA.

rellevants de forma consecutiva.

5.5. REVERSE TRANSCRIPTION

RT is performed in order to obtain cDNA from purified miRNA and the reaction is catalyzed by reverse transcriptase. Although mRNAs are polyadenylated, miRNAs are not. miRNAs need to be polyadenylated by means of poly(A) polymerase, (see Figure 7 for details). Both reactions occur in the same tube.

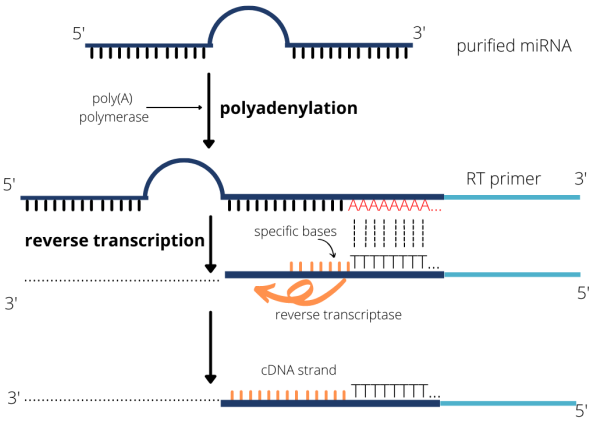

Figure 7. Reverse transcription and polyadenylation

Total RNA was reverse transcribed into cDNA following the manufacturer's protocol (miRCURY LNA miRNA PCR protocol).

While using an RNA carrier, it is not possible to know the exact concentration of RNA after purification. Therefore, the volume is based of input RNA for the RT reaction on the original volume of the starting sample material. All reactions are performed on ice in order to minimize the risk of RNA degradation. dNTPs are already included in the kit components. It is also used the RNA Spike-in, which is a synthetic RNA that identifies instrument or chemistry failures, errors in assay setup or the presence of inhibitors as some samples may contain compounds that inhibit the cDNA synthesis or the PCR even though the RNA has been purified. It is added 1 µl of synthetic spike-in UniSP6 (108 copies/µl) per 20 ng sample RNA. The 10x miRCURY RT Enzyme Mix includes Poly(A) polymerase and reverse transcriptase. The RT primer is included in the 5x miRCURY RT SYBR ® Green Reaction Buffer.

The mix contains: 2 µl of 5x miRCURY SYBR ® Green RT Reaction Buffer, 4,9 µl of RNase-free water, 1 µl of 10x miRCURY RT Enzyme Mix, 0,5 µl UniSp6 RNA spike-in (control) and 1,6 µl of template RNA. It is prepared each volume per 10 samples and then it is mixed in each tub 8,4 µl of mix and 1,6 µl of RNA of each sample for a total volume of 10 µl.

It is also prepared a mix for blank reagent which contains: 2 µl of 5x miRCURY SYBR ® Green RT Reaction Buffer, 4,9 µl of RNase-free water and 4 µl of 10x miRCURY RT Enzyme Mix. It is prepared a solution of MS2 100 µg/ml diluting with RNase-free water a solution of MS2 0,8 µg/ml. It is mixed in each tub 8,4 µl of mix and 1,6 µl of MS2 100 µg/ml.

Table 1. Kit conditions (time, temperature) of the RT reaction according to the manufacture's protocol

Entry	Step	Time	Temperature
1	Reverse-transcription step	60 min	42ºC
2	Inactivation of reaction	5 min	95ºC
3	Storage	∞	4ºC

When all the reagents are mixed, tubes are placed in the thermocycler Thermal Cycler by thermo Fischer scientific SimpliAmp™ and the proper cycles are programmed according to the

manufacture's protocol (see Table 1 for details). The first step is the incubation for 60 min at 42ºC, followed by the incubation for 5 min at 95ºC to heat inactivate the reverse transcriptase; finally, the mix is placed on an ice-bath. Tubes can be stored undiluted at 2–8ºC for up to 4 days or at -30 to -15ºC for up to 5 weeks if the cDNA is not used immediately.

5.6. REAL-TIME PCR

qPCR is a rapid and sensitive technique performed in molecular biology to obtain several copies of small sequences of DNA or a gene. With PCR it is possible to obtain thousands to millions of copies of a few copies of DNA.

To perform this technique, some reagents are needed. First of all, the DNA template that in this study, is the one obtained after the RT reaction. Primers are also needed; they are short sequences of DNA that initiate the qPCR reaction, designed to bind to either side of the interest section of DNA, DNA nucleotides (dNTPs), corresponding to the four DNA bases (A, C, G and T), and a polymerase, which adds new DNA bases.

General qPCR mechanism includes three steps, as we observe in figure 8, which are detailed below.

First of all, the double-stranded DNA is denatured by heating over 90ºC. The high temperature breaks the hydrogen bonds between the bases of the two strands and two single strands are obtained that act as templates for the synthesis of the new strands of DNA. The annealing step occurs by cooling to 50-65ºC. This temperature, which depends on the melting temperature of the primers, is needed to the primers to be complementary in sequence of interest on the single-stranded template DNA by hydrogen bonding. In the last step, extending, the temperature is increased to 72ºC and the new strand of DNA is synthesized by the polymerase enzyme, in these case, QuantiNova DNA Polymerase. It usually takes around one minute to copy 1000 DNA bases (1Kb). These three stages are repeated 40-45 times normally, doubling the number of DNA copies each time.

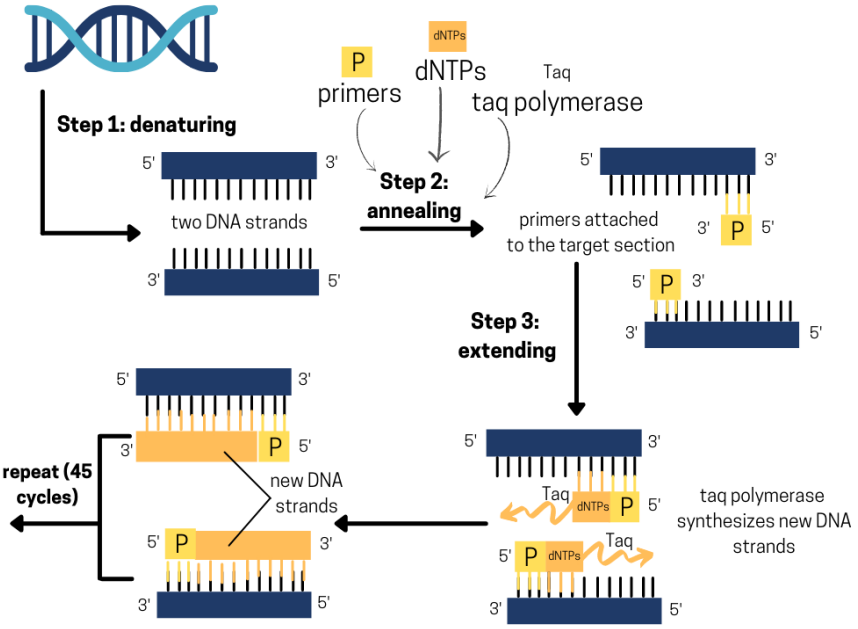

Figure 8. Mechanism of the PCR

The first step in the qPCR procedure is to dilute the cDNA 1:30 with RNase-free water. Two mixes are then prepared: primer mix and UniSp6 mix. The primer mix is prepared for the 10 samples and 1 MS2 sample. MS2 acts as a blank reagent, showing no fluorescence in the melting curves. UniSp6 amplifies around 15 cycles.

The primer mix contains 5 µl of 2x miRCURY SYBR® Green Master Mix, 1 µl resuspended PCR primer mix, and 1 µl of RNase-free water. It will be used 7 µl of the mix per qPCR plate and then is added 3 µl of cDNA template in each plate.

The UniSp6 mix contains: 5 µl 2x miRCURY SYBR® Green Master Mix, 1 µl Primer UniSp6, and 1 µl of RNase-free water. It will be used 7 µl of the mix per qPCR plate and then is added 3 µl of cDNA template in each plate.

Data are obtained while the qPCR reaction takes place, hence becoming amplification and detection into a single step. The technique is performed through fluorescent chemistries that relate qPCR product concentration to fluorescence intensity. Reactions are characterized by a value called cycle threshold (C_t) when the target amplification is first detected, at the time at

which fluorescence intensity is higher than background fluorescence. As a result, when there is a higher concentration of target DNA in the starting material, the fluorescent signal will be increased faster, occurring thus at a lower C_t.[34]

At first, the qPCR was programmed according to the conditions of the manufacture's protocol, as we observe in the Table 2. Nevertheless, as we see in figure 9, no amplification did happen because the cycles of the melting curves obtained were impossible to interpretate.

Table 2. Kit conditions (time, temperature, number of cycles and ramp rate) of the PCR according to the manufacture's protocol

Entry	Step	Time	Temperature	Number of cycles	Ramp rate
1	Heat activation	2 min	95ºC	1 cycle	4,4 ºC/s
2	Denature	10 seg	95ºC	1 cycle	4,4 ºC/s
3	Amplification	1 min	56ºC	45 cycles	2,2 ºC/s
4	Melting	1 seg	60ºC	1 cycle	4,4 ºC/s
5		1 seg	95ºC		4,4 ºC/s
6	Cooling	1 min	40ºC	1 cycle	2,2 ºC/s

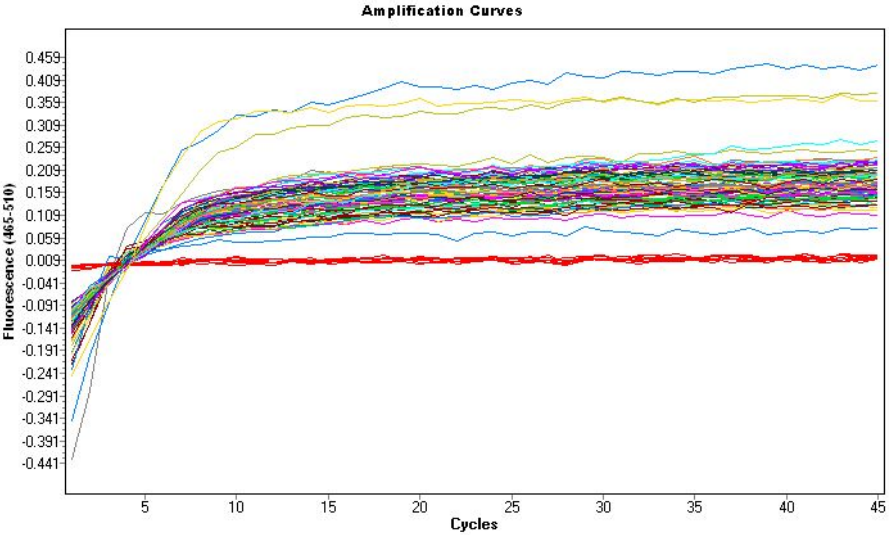

Figure 9. Amplification curves obtained following the kit's conditions of temperature, number of cycles and ramp rate in PCR

Thereby, some conditions were adjusted as we observe in Table 3. We could see correct amplification curves, as it is showed in figure 10, and cycles of the melting curves were possible to interpretate. UniSp6 is amplified at around 15 cycles, and different colored lines correspond to each sample. MS2 as a blank reagent, showed no amplification (no fluorescence detected).

Table 3. Modifications in the conditions (time, temperature, number of cycles and ramp rate) in the manufacture's protocol

Entry	Step	Time	Temperature	Number of cycles	Ramp rate
1	No heat activation	-	-	-	-
2	Denature	2 min	95ºC	1 cycle	4,4 ºC/s
3	Amplification	10 seg	95ºC	45 cycles	4,4 ºC/s
4		1 min	56ºC		1,6 ºC/s
5	Melting	Continuous	95ºC	1 cycle	0,11 ºC/s
6	Cooling	1 min	40ºC	1 cycle	2,2 ºC/s

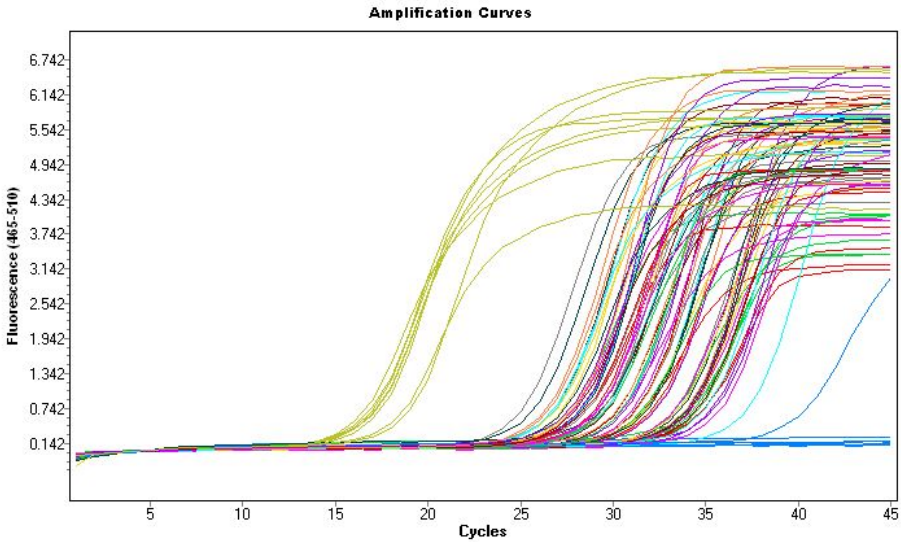

Figure 10. Amplification curves obtained following the modifications stablished in the conditions (time, temperature, number of cycles and ramp rate)

According to the Technical note No. LC 13/2001 from Roche®, the easiest way to obtain a quantitative value for an unknown concentration of a target is to use external standards or calibrator. The expression of the results are thus a ratio between target and reference. There are several mathematical models available to calculate the mean normalized gene expression from relative quantitation assays, however in this study, relative expression of miRNAs is obtained by the formula $2^{-\Delta Ct}$.

The amplification curves were analyzed using Roche LC Software for obtaining the value of C_t by the second derivative method. Following the manufacturer's instructions, ΔC_t was calculated as the difference in C_t values between the miRNA target and the mean of reference control (miR-191-5p and miR-103-5p). Relative expression levels of target miRNAs are calculated by $2^{-\Delta Ct}$, where high values refer to a higher expression of miRNAs.

5.7. STATISTICAL ANALYSIS

All the analyses are performed by SPSS 23.0 software. After obtaining miRNAs expression, values from the different groups were compared using the Mann-Whitney test. Receiver operating characteristic curve (AUROC) with 95% confidence is also performed in order to stablish optimal cut-off points.

6. RESULTS AND DISCUSSION

6.1. METHOD STANDARDIZATION

For miRNAs analysis, qPCR conditions were optimized as mentioned in section 5.6. An intra and inter-assay were performed in order to stablish coefficient of variation (%CV) of the samples.

The performance of the intra-assay was based on a qPCR panel for two cDNA samples which were mixed with the necessary reagents for the reaction (see section 5.). Both two samples (FIS23V7 and FIS50V3) were pipetted 4 times for miR155, miR122, miR181a, miR191, mir103 and UniSp6. It was also pipetted MS2 as blank reagent.

The results obtained from the intra-assay of the two samples are shown in Table 4 and 5. Results from Table 4 show a %CV of 6.32, 3.95, 4.69, 4.55 and 2.18 for miRNA-155-5p, miRNA-122-5p, miRNA-181-5p, miRNA-191 and miRNA-103, respectively. Results from Table 5, show a %CV of 3.64, 6.57, 5.04, 3.77, 2.44 for miRNA-155-5p, miRNA-122-5p, miRNA-181-5p, miRNA-191 and miRNA-103, respectively. All %CV values are below 7%, so it is considered a low dispersion for each miRNA.

Table 4. Results of intra-assay (mean Ct, standard deviation, relative value, and precision of the method) for sample FIS23V7

	miRNA-155	miRNA-122	miRNA-181	miRNA-191	miRNA-103
Mean C_t day 1	34.37	30.26	32.55	28.32	28.30
Std dev	2.17	1.20	1.53	1.29	0.62
$2^{-\Delta Ct}$	0.015	0.258	0.030	-	-
%CV	6.32	3.95	4.69	4.55	2.18

(a) The mean of Ct corresponds to the mean of the Ct values obtained after PCR for each sample in 4 independent assays performed in parallel
(b) Standard deviation of the Ct values for each sample was also calculated.
(c) The relative values were calculated by the formula 2-DCt
(d) CV: coefficient of variation

Table 5. Results of intra-assay (mean Ct, standard deviation, relative value, and precision of the method) for sample FIS50V3

	miRNA-155	miRNA-122	miRNA-181	miRNA-191	miRNA-103
Mean C_t day 1	35.54	30.44	31.74	29.18	28.71
Std dev	1.29	2	1.6	1.1	0.7
$2^{-\Delta Ct}$	0.010	0.355	0.144	-	-
%CV	3.64	6.57	5.04	3.77	2.44

(a) See table 4 legend for details

It was performed an inter-assay, where the results of both samples (FIS23V7 and FIS50V3) were compared to the results of the same samples respectively, performed another day.

The results obtained from the inter-assay are shown in Table 6 and 7 below respectively for each sample. Table 6 show a %CV of 2.68, 3.87, 3.02 and 1.21; and an exactitude of 0.039,

0.056, 0.044, 0.017 and 0.027 for miRNA-155-5p, miRNA-122-5p, miRNA-181-5p, miRNA-191 and miRNA-103, respectively. Table 7 show a %CV of 3.3, 1.98, 4.17, 0.29 and 0.15; and an exactitude of 0.048, 0.028, 0.061, 0.004 and 0.002 for miRNA-155-5p, miRNA-122-5p, miRNA-181-5p, miRNA-191 and miRNA-103, respectively.

For both samples, %CV are below 4%, so their dispersion is low, as a %CV below 10 for biological samples is accepted. The exactitude is below 0.1 for both samples, so the method is considered exact with acceptable criteria to its implement in routine.

Table 6. Results of inter-assay (precision and exactitude of the method) for sample FIS23V7.

	miRNA-155	miRNA-122	miRNA-181	miRNA-191	miRNA-103
Mean C_t day 1	34.37	30.26	32.55	28.32	28.30
Mean C_t day 2	33.09	28.65	31.19	28.81	29.08
Mean C_t day 1 and day 2	33.73	29.46	31.87	28.57	28.69
Std. dev. day 1 and day 2	0.91	1.14	0.96	0.35	0.55
%CV	**2.68**	**3.87**	**3.02**	**1.21**	**1.92**
Exactitude	**0.039**	**0.056**	**0.044**	**0.017**	**0.027**

(a) The analysis was repeated another day (mean Ct day 2).
(b) Mean Ct and standard deviation between day 1 and day 2 was then calculated.
(c) The precision of the method was calculated between mean Ct and the standard deviation from day 1 and 2.
(d) The exactitude of the method was calculated between mean Ct day 1 and day 2.

Table 7. Results of inter-assay (precision and exactitude of the method) for sample FIS50V3.

	miRNA-155	miRNA-122	miRNA-181	miRNA-191	miRNA-103
Mean C_t day 1	35.54	30.44	31.74	29.18	28.71
Mean C_t day 2	33.92	29.6	29.92	29.3	28.77
Mean C_t day 1 and day 2	34.73	30.02	30.83	29.24	28.74
Std. dev. day 1 and day 2	1.15	0.59	1.29	0.08	0.04
%CV	3.3	1.98	4.17	0.29	0.15
Exactitude	0.048	0.028	0.061	0.004	0.002

a) See legend of Table 6 for details.

6.2. CLINICAL RESULTS

During my stay of 4 months in the Laboratory of Pharmacology and Toxicology, I have analyzed samples from 25 patients, 12 were follow-ups and 13 were new liver-transplanted patients. Four patients presented TCMAR (biopsy proven acute rejection), 2 of them 1 week after LT, which were follow-ups, and 2 of them 15 days after LT, which were new liver-transplanted patients. One of new liver-transplanted patients was exitus due to septic shock with multi-organ failure.

6.3. MIRNA RESULTS

Data were adjusted to a non-parametrical distribution, and mean, median and deviation are calculated for each miRNA in non-rejector patients (Tables 8 to 13) and in TCMAR patients (Tables 14 and 15) after LT among 6 months. Table 8 show a miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p median of 0.067, 1.224 and 0.216, respectively; Table 9, 0.056, 1.079 and 0.208; Table 10, 0.033, 1.080 and 0.203; Table 11, 0.027, 0.632 and 0.153; Table 12 0.032, 0.578 and 0.247; Table 13, 0.042 0.616 and 0.390; Table 14, 1.524, 7.261 and 1.635; and finally, Table 15, 2.077, 9.160 and 1.978. As we can observe, in Table 14 and 15, miRNAs median is higher than the other tables; so rejector patients present higher miRNAs than non-rejector.

Table 8. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients the first week after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.149	1.668	0.374
Median	**0.067**	**1.224**	**0.216**
Std. Dev.	0.192	1.602	0.373
Minimum	0.008	0.062	0.022
Maximum	0.653	7.135	1.218

Table 9. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients 15 days after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.071	1.354	0.347
Median	**0.056**	**1.079**	**0.208**
Std. Dev.	0.068	1.018	0.302
Minimum	0.006	0.031	0.077
Maximum	0.265	3.053	1.193

Table 10. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients 1 month after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.046	1.174	0.302
Median	**0.033**	**1.080**	**0.203**
Std. Dev.	0.039	0.972	0.260
Minimum	0.006	0.035	0.051
Maximum	0.154	2.969	1.042

Table 11. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients 2 months after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.041	0.870	0.234
Median	**0.027**	**0.632**	**0.153**
Std. Dev.	0.040	0.677	0.221
Minimum	0.009	0.009	0.069
Maximum	0.164	2.282	0.933

Table 12. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients 3 months after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.042	0.618	0.237
Median	**0.032**	**0.578**	**0.247**
Std. Dev.	0.053	0.391	0.122
Minimum	0.006	0.005	0.079
Maximum	0.210	1.464	0.535

Table 13. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for non-rejector patients 6 months after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	0.056	0.893	0.681
Median	**0.042**	**0.616**	**0.390**
Std. Dev.	0.030	0.749	0.560
Minimum	0.035	0.260	0.092
Maximum	0.119	2.092	1.619

Table 14. Results of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p for TCMAR patients 1 week after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	1.574	6.899	3.339
Median	**1.524**	**7.261**	**1.635**
Std. Dev.	0.642	2.518	3.762
Minimum	1.013	3.880	1.147
Maximum	2.235	9.193	8.940

Table 15. Results of miRNA-155-5p. miRNA-122-5p and miRNA-181a-5p for TCMAR patients 15 days after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mean	2.077	9.160	1.978
Median	**2.077**	**9.160**	**1.978**
Std. Dev.	0.005	6.433	0.490
Minimum	2.073	4.611	1.631
Maximum	2.080	13.708	2.324

Figures 11, 12 and 13 summarize all the obtained results for miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p, respectively for patients with TCMAR compared with those patients free of rejection.

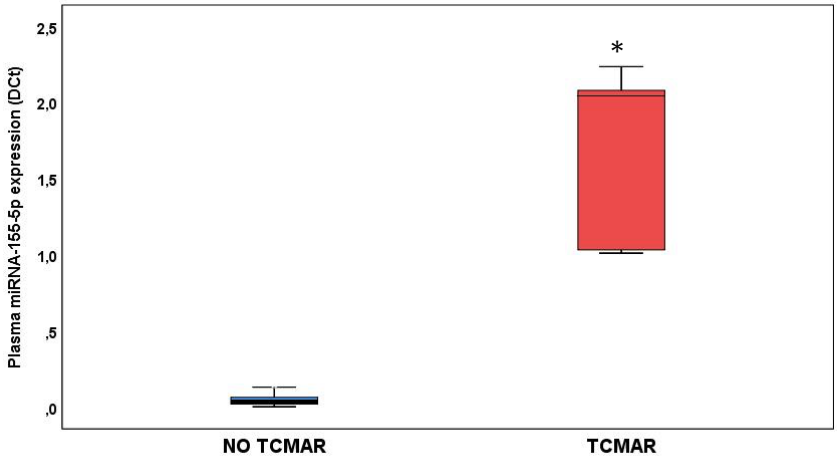

Figure 11. miRNA-155-5p expression for non-rejector patients and for TCMAR patients.

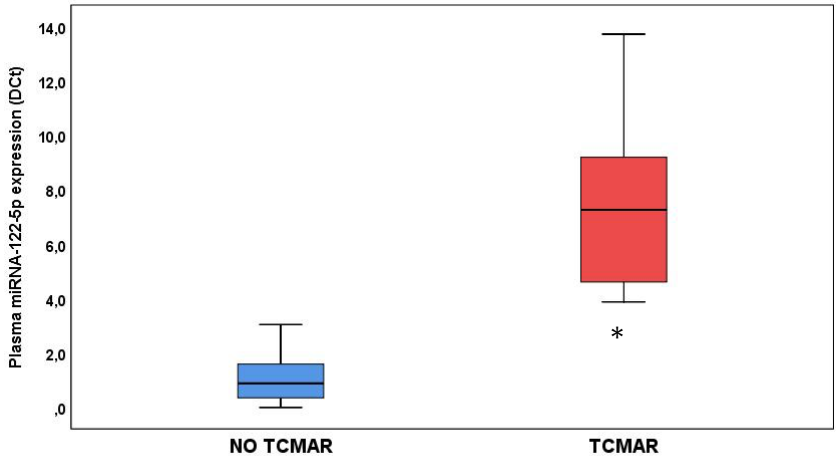

Figure 12. miRNA-122-5p expression for non-rejector patients and for TCMAR patients

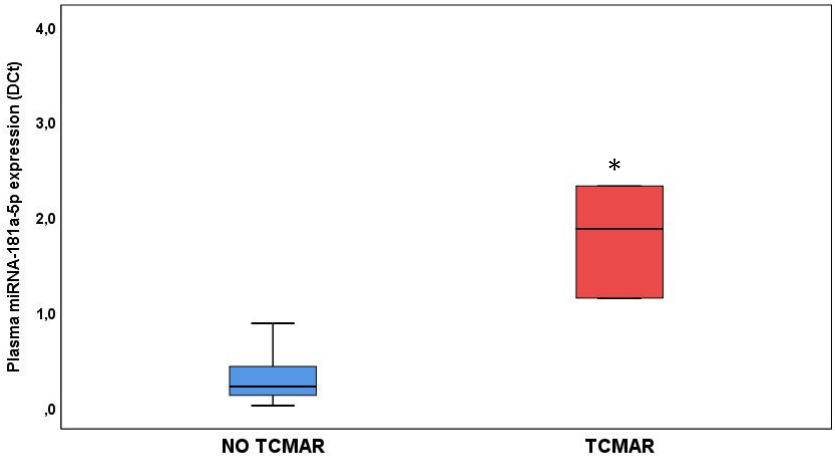

Figure 13. miRNA-181a-5p expression for non-rejector patients and for TCMAR patients

In order to compare non-rejector and TCMAR patients, it is evaluated not only the diagnostic capacity of these biomarkers but their predictive potential. Box-plot graphics (Figures 14, 15 and

16), which correspond to the frequency of monitoring of these biomarkers during the study period, are included. In these figures, the first week after LT there were 4 patients with this clinical event, 2 patients with biopsy proven TCMAR and 2 patients at a high risk of rejection with high expression of miRNAs that finally rejects 15 days after LT. During the next months, no rejection occurred as the treatment was modified to the rejector patients. All patients free of rejection have miRNAs expressions under the stablished cut-off value (black line) during all the period of the study.

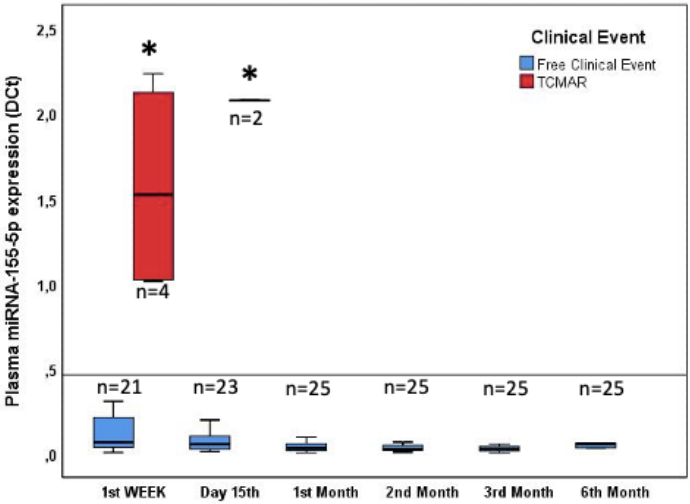

Figure 14. Comparation of plasma miRNA-155-5p expression in TCMAR patients and in non-rejector patients over 6 months after LT ($P<0.05$). Cut-off = 0.450.

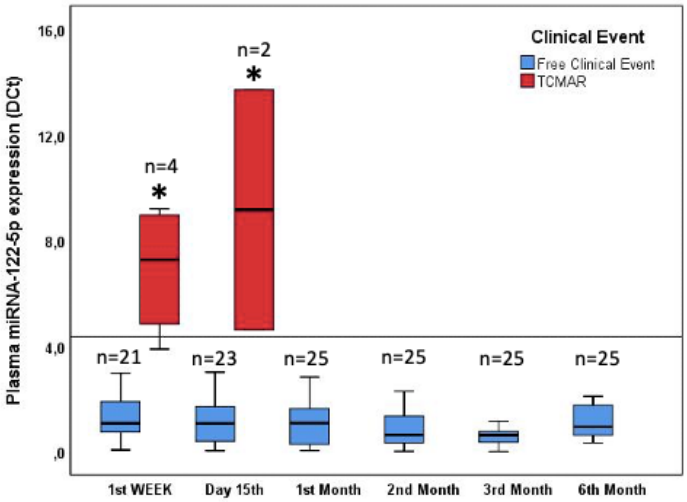

Figure 15. Comparation of plasma miRNA-122-5p expression in TCMAR patients and in non-rejector patients over 6 months after LT (P<0.05). Cut-off = 4.246.

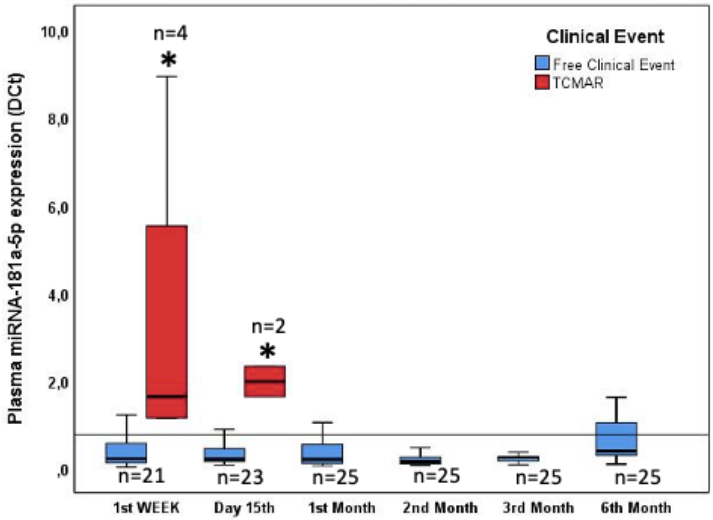

Figure 16. Comparation of plasma miRNA-181a-5p expression in TCMAR patients and in non-rejector patients over 6 months after LT (P<0.05). Cut-off = 0.763.

Mann-Whitney test evaluate statistical differences between non-rejector and TCMAR patients. Exact signification (P) is obtained in this test, and this value expresses if considerable differences are shown in both groups. For the first week after LT, exact signification between rejectors and non-rejectors is 0 for miRNA-155-5p; 0.001 for miRNA-122-5p; and 0.001 for miRNA-181a-5p (see Table 16 for details). For the first 15 days after LT, exact signification between rejectors and non-rejectors is 0.008 for miRNA-155-5p; 0.008 for miRNA-122-5p; and 0.008 for miRNA-181a-5p. (see Table 17 for details). We have obtained $P<0.05$, which is considered statistically significant, so both groups can be differentiated by these biomarkers.

Table 16. Mann-Whitney test results showing statistical differences between rejectors and non-rejectors first week after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mann-Whitney U	0	2	2
Wilcoxon W	171	173	173
Z	-3.065	-2.894	-2.894
asymptotic signification	0.002	0.004	0.004
exact signification (P)	**0.000**	**0.001**	**0.001**

Table 17. Mann-Whitney test results showing statistical differences between rejectors and non-rejectors 15 days after LT

	Plasma miRNA-155-5p expression (DCt)	Plasma miRNA-122-5p expression (DCt)	Plasma miRNA-181a-5p expression (DCt)
Mann-Whitney U	0	0	0
Wilcoxon W	231	231	231
Z	-2.292	-2.291	-2.291
asymptotic signification	0.022	0.022	0.022
exact signification (P)	**0.008**	**0.008**	**0.008**

Afterwards, ROC curve analysis (Figures 14, 15 and 16) with 95% confidence, differentiates both groups and stablishes the optimal cutoff value, which is obtained by the optimal Youden

index (sensitivity + specificity – 1). The area under the curve (AUC) stablishes the discriminatory capacity of miRNAs, being >0,9 an excellent discrimination between groups. For miRNA-155-5, AUC is 1 and cutoff point is 0.45; for miRNA-122-5p, AUC is 0.986 and cutoff point is 4.246; and for miRNA-181a-5p, AUC is 0.99 and cut off point is 0.763. AUC obtained for each miRNA are higher than 0.9 so these biomarkers can discriminate rejector from non-rejector patients, being miRNA-155-5p the best of them.

These results in this independent cohort of adult liver transplanted recipients demonstrate the potential of the evaluated miRNAs as predictive biomarkers of TCMAR, and strongly corroborates results from previous study involving patients with similar characteristics[6]. Even more, the cut-off values stablished for each miRNA were practically the same. For miRNA-155-5p, cut-off obtained is 0.45 with a sensitivity of 100% and specificity of 98%; while in previous study was 0.463 with a sensitivity of 91% and specificity of 95%. For miRNA-122-5p, cut-off obtained is 4.246 with a sensitivity of 83% and specificity of 98%; while in previous study was 4.356 with a sensitivity of 93% and specificity of 90%. For miRNA-181a-5p, cut-off obtained is 0.76 with a sensitivity of 90% and specificity of 95%; while in previous study was 0.763 with a sensitivity of 100% and specificity of 83%.

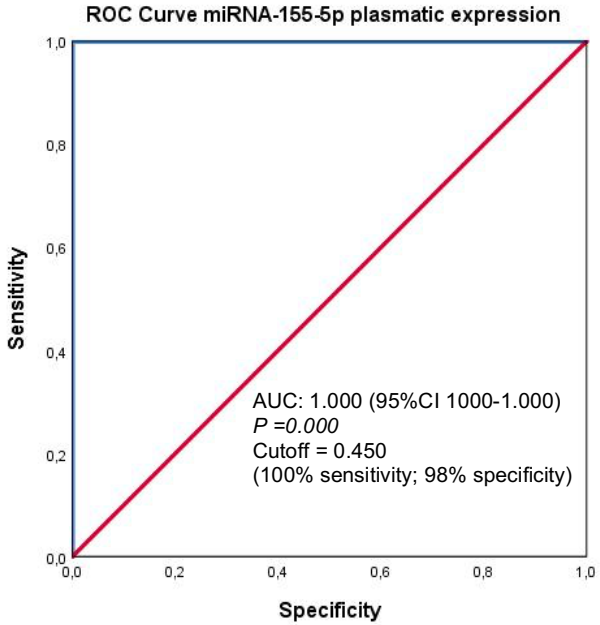

Figure 14. ROC curve for TCMAR diagnosis for miRNA-155-5p plasmatic expression

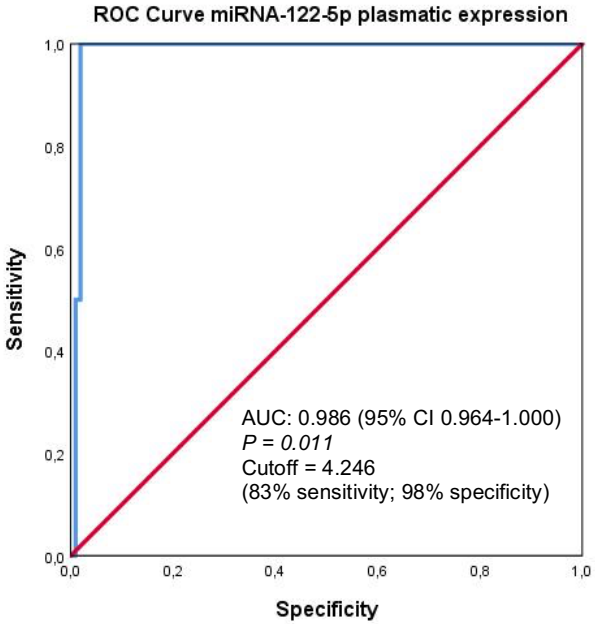

Figure 15. ROC curve for TCMAR diagnosis for miRNA-122-5p plasmatic expression

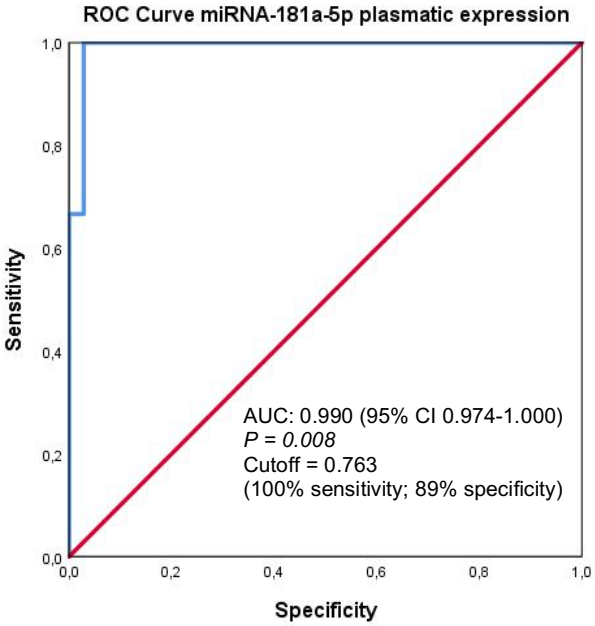

Figure 16. ROC curve for TCMAR diagnosis for miRNA-181a-5p plasmatic expression

Table 18. AST, ALT, alkaline phosphatase and GGT concentration for the patients suffering TCMAR after LT

	AST (IU/L)	ALT (IU/L)	Alkaline phosphatase (IU/L)	GGT (IU/L)
Patient 1, first week	135	535	174	613
Patient 2, first week	111	821	69	190
Patient 3, first week	35	345	165	285
Patient 3, first 15 days	138	290	589	866
Patient 4, first week	36	52	90	131
Patient 4, first 15 days	61	52	111	243

AST, ALT, alkaline phosphatase and GGT are conventional biomarkers which are controlled in transplanted patients to prevent AR. Table 18 show these values for the four patients who suffered TCMAR, where two of them manifested TCMAR the first week after LT, and two of them manifested TCMAR the first 15 days after LT. AST, ALT, alkaline phosphatase and GGT levels in serum, are 135, 535, 174 and 613; and 111, 821, 69 and 190 IU/L respectively, for the two patients who manifested TCMAR the first week after LT. For the two patients who manifested TCMAR the first 15 days after LT, showed AST, ALT, alkaline phosphatase and GGT levels in serum the first week after LT of 35, 345, 165 and 285; and 36, 52, 90 and 131 IU/L, respectively. These two patients presented 36, 52, 90 and 131; 61, 52, 111 and 243 IU/L for these biomarkers, at the time when they manifested TCMAR (next 15 days after LT). 10-40 IU/L are normal values for adults for ALT in serum; 8 to 40 IU/L for AST; 44 to 147 IU/L for alkaline phosphatase; and 7 to 55 IU/L for GGT. For one patient who manifested TCMAR the first week after LT, alkaline phosphatase level is within the normal range. For one patient who manifested TCMAR the first 15 days after LT, AST level is within the normal range, the first week after LT. For another patient who manifested TCMAR the first 15 days after LT, AST, ALT and alkaline phosphatase are within the normal range, or are not too high from the range. As we can observe, these biomarkers were able to predict TCMAR in two out of four patients with TCMAR. Thus, this biochemical results clearly show the requirement for more sensitive and specific noninvasive biomarkers to better predict TCMAR.

7. CONCLUSIONS

The present study shows that a panel of miRNAs (miRNA-155-5p, miRNA-181a-5p and miRNA-122-5p) is able to predict TCMAR in adult liver transplanted recipients.

miRNA-155-5p has been identified as the best predictor of the rejection process with a cut-off value of 0.45 with a sensitivity of 100% and specificity of 98%.

Serum levels of miRNA-155-5p, miRNA-122-5p and miRNA-181a-5p are increased before the diagnosis of TCMAR by biopsy, thus showing its potential predictive value.

miRNAs can diagnose in a more specific and sensitive way allograft rejection than conventional biochemical biomarkers currently implemented in clinical routine.

The results obtained in this independent cohort of adult liver transplanted recipients, are in agreement with those previously observed in a population with similar demographic and clinical characteristics. But confirmation by other centers in the context of multicentric studies involving a high number of patients is needed.

8. REFERENCES AND NOTES

1. European Association for the Study of Liver. EASL Clinical Practice Guidelines: Liver transplantation. J Hepatol. 2016, 64 (2), 433-485.
2. Krenzien, F.; Keshi, E.; Splith, K.; et al. Diagnostic Biomarkers to Diagnose Acute Allograft Rejection After Liver Transplantation: Systematic Review and Meta-Analysis of Diagnostic Accuracy Studies. Front Immunol. 2019, 10, 758.
3. Charlton, M.; Levitsky, J.; Aqel, B.; et al. International Liver Transplantation Society Consensus Statement on Immunosuppression in Liver Transplant Recipients. Transplantation. 2018, 102 (5), 727-743.
4. Gelson, W.; Hoare, M; Dawwas, M.F.; et al. The pattern of late mortality in liver transplant recipients in the United Kingdom. Transplantation. 2011, 91 (11), 1240-1244.
5. Wang, W.; Wang, B.; Liu, C.; et al. Serum proteomic predicts effectiveness and reveals potential biomarkers for complications in liver transplant patients. Aging. 2020, 12 (12), 12119-12141.
6. Millán, O.; Ruiz, P.; Orts, L.; et al. Monitoring of miR-181a-5p and miR-155-5p Plasmatic Expression as Prognostic Biomarkers for Acute and Subclinical Rejection in de novo Adult Liver Transplant Recipients. Front Immunol. 2019, 10, 873.
7. Ronca, V.; Wootton, G.; Milani, C.; Cain, O. The Immunological Basis of Liver Allograft Rejection. Front Immunol. 2020, 11.
8. Choudhary, N. S.; Saigal, S; Bansal, R. K.; et al. Acute and Chronic Rejection After Liver Transplantation: What A Clinician Needs to Know. J Clin Exp Hepatol. 2017, 7 (4), 358.
9. Seyfinejad, B.; Jouyban, A. Overview of therapeutic drug monitoring of immunosuppressive drugs: Analytical and clinical practices. J Pharm Biomed Anal. 2021, 205.
10. Pirmohamed, M. Pharmacogenetics and pharmacogenomics. Br J Clin Pharmacol. 2001, 52 (4), 345.
11. García-González, X.; Cabaleiro, T.; Herrero, M. J.; et al. Clinical implementation of pharmacogenetics. Drug Metab Pers Ther. 2016, 31 (1), 9-16.
12. Brunet, M; van Gelder, T.; Åsberg, A.; et al. Therapeutic Drug Monitoring of Tacrolimus-Personalized Therapy: Second Consensus Report. Ther Drug Monit. 2019, 41 (3), 261-307.
13. Toby, T. K.; Abecassis, M.; Thomas, P. M; et al. Proteoforms in Peripheral Blood Mononuclear Cells as Novel Rejection Biomarkers in Liver Transplant Recipients. Am J Transplant. 2017, 17 (9), 2458-2467.
14. Germani, G.; Rodriguez-Castro, K.; Russo, F. P.; et al. Markers of acute rejection and graft acceptance in liver transplantation. World J Gastroenterol. 2015, 21 (4), 1061-1068.
15. Conti, F.; Frappier, J.; Dharancy, S.; et al. Interleukin-15 production during liver allograft rejection in humans. Transplantation. 2003, 76 (1), 210-216.
16. Millán, O; Brunet, M. Cytokine-based immune monitoring. Clin Biochem. 2016, 49 (4-5), 338-346.
17. Millán, O.; Budde, K.; Sommerer, C. et al. Urinary miR-155-5p and CXCL10 as prognostic and predictive biomarkers of rejection, graft outcome and treatment response in kidney transplantation. Br J Clin Pharmacol. 2017, 83 (12), 2636-2650.
18. Gielis E. M.; Anholts, J. D. H.; van Beelen, E.; et al. A Combined microRNA and Chemokine Profile in Urine to Identify Rejection After Kidney Transplantation. Transplant direct. 2021, 7 (7).
19. Raschzok, N.; Reutzel-Selke, A.; Schmuck, R. B.; et al. CD44 and CXCL9 serum protein levels predict the risk of clinically significant allograft rejection after liver transplantation. Liver Transpl. 2015, 21 (9), 1195-1207.

20. Friedman, B. H.; Wolf, J. H.; Wang, L.; et al. Serum cytokine profiles associated with early allograft dysfunction in patients undergoing liver transplantation. Liver Transpl. 2012, 18 (2), 166-176.
21. Zhang, K.; Sun, Y.L.; Zhou, S. N.; et al. Circulating CXCR3-CCR6-CXCR5+CD4+ T cells are associated with acute allograft rejection in liver transplantation. Immunol Lett. 2019, 213, 55-61.
22. Tinel, C.; Lamarthée, B.; Anglicheau, D. MicroRNAs: small molecules, big effects. Curr Opin Organ Transplant. 2021, 26 (1), 10-16.
23. Lee, R. C.; Feinbaum, R. L.; Ambros, V. The C. Elegans Heterochronic Gene Lin-4 Encodes Small RNAs with Antisense Complementarity to Lin-14. Cell. 1993, 75 (5), 843–854.
24. Wightman, B.; Ha, I.; Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993, 75 (5), 855-862.
25. Harris, A.; Krams, S. M.; Martinez, O. M. MicroRNAs as Immune Regulators: Implications for Transplantation. Am J Transplant. 2010, 10 (4), 713.
26. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol. 2018, 9, 402
27. Hamdorf, M.; Kawakita, S.; Everly, M. The Potential of MicroRNAs as Novel Biomarkers for Transplant Rejection. J Immunol Res. 2017, 4072364..
28. Steer, C. J.; Subramanian, S. Circulating microRNAs as biomarkers: a new frontier in diagnostics. Liver Transpl. 2012, 18 (3), 265-269.
29. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004, 116 (2), 281-297.
30. Saliminejad, K.; Khorshid, H. R. K.; Fard, S. S.; Ghaffari, S. H. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. J Cell Physiol. 2019, 234 (5), 5451-5465.
31. Raisch, J.; Darfeuille-Michaud, A.; Nguyen, H. T. T. Role of microRNAs in the immune system, inflammation and cancer. World J. Gastroenterol. 2013, 19 (20), 2985-2996.
32. Bernardo, B.; Ooi J.; Lin, R.; et al. miRNA therapeutics: a new class of drugs with potential therapeutic applications in the heart. Future medicinal chemistry. 2015, 7 (13), 1771-1792.
33. Sayed, D.; Abdellatif, M. MicroRNAs in development and disease. Physiol Rev. 2011, 91 (3), 827-887.
34. Donati, S.; Ciuffi, S.; Brandi, M. L. Human Circulating miRNAs Real-time qRT-PCR-based Analysis: An Overview of Endogenous Reference Genes Used for Data Normalization. Int J Mol Sci. 2019, 20 (18).
35. Ruiz, P.; Millán, O.; Ríos, J.; et al. MicroRNAs 155-5p, 122-5p, and 181a-5p Identify Patients With Graft Dysfunction Due to T Cell-Mediated Rejection After Liver Transplantation. Liver Transpl. 2020, 26 (10), 1275-1286.
36. Waqar, R. R. F.; Pan, Q.; van der Meer, A. J. P.; et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl. 2012, 18 (3), 290-297.
37. Di Maira, T.; Little, E. C.; Berenguer, M. Immunosuppression in liver transplant. Best Pract Res Clin Gastroenterol. 2020, 46-47.
38. Chooi, Y.; Tang, Y. Navigating the Fungal Polyketide Chemical Space: From Genes to Molecules. J Org Chem. 2012, 77 (22), 9933.
39. Zhang M.; Tajima S.; Shigematsu T.; et al. Donor CYP3A5 Gene Polymorphism Alone Cannot Predict Tacrolimus Intrarenal Concentration in Renal Transplant Recipients. Int J Mol Sci. 2020, 21 (8), 2976.
40. Tasdogan, B. E.; Ma, Mi.; Simsek, C.; et al. Update on Immunosuppression in Liver Trasplantation. Euroasian J Hepatogastroenterol. 2019, 9 (2), 96-101.

9. ACRONYMS

IS: immunosuppressive

LT: liver transplantation

AR: allograft rejection

SCR: inflammatory lesions in the allograft

TCMAR: acute T-cell mediated rejection

ABMR: antibody mediated rejection

PK: pharmacokinetic

PD: pharmacodynamic

TDM: therapeutic drug monitoring

ALT: alanine aminotransferase

AST: aspartate aminotransferase

GGT: γ-glutamyl transpeptidase

NFAT: nuclear factor of activated t-cell regulated transcription factor

AGO2: argonaute2 protein

RISC: miRNA-induced silencing complex

qPCR: real time quantitative polymerase chain reaction

TGF-ß: transforming growth factor beta

CNI: calcineurin inhibitors

Tac: tacrolimus

LNA: locked nucleic acids

RT: reverse transcription

Ct: cycle threshold

CV: coefficient of variation