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CICLO **XXX**

THESIS TITLE:

Identification of molecular biomarkers to discriminate and characterize the different types of rejection in Heart Transplated Patients

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Abbreviations.

Ab/Abs	Antibody/Antibodies
ACR	Acute Cellular Rejection
AMR/pAMR	Antibody Mediated Rejection
α-SMA	Smooth muscle actin
BSA	Bovine serum albumin
CD3	Cluster Differntiation 3
CD68	Cluster Differentiation 68
Cy3	Cyanine 3
Cy5	Cyanine 5
DAPI	4',6-Diamidino-2-phenylindol
DIG	Digoxigenin
DSA	Donor Specific Antibodies
dsRNA	Double-stranded RNA
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EMB/EMBs	Endomyicardial biopsy
FFPE	Formalin Fixed Paraffin Embedded
FITC	Fluorescein isothiocyanate
Hsa	Homo sapiens
IL-1	Interleukin 1
IL-6	Interleukin 6
IP	ImmunoPrecipitation
MAPK	mitogen-activated protein kinase
Min	Minute
MiR, MiRNA	MicroRNA
MiRISC	MiRNA induced silencing complex
MIX	Mixed Rejection

NF-kB	Nuclear factor kappa B
ON	Overnight
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
qRT-PCR	Quantitative real-time polymerase chain reaction
RNU46	Small nucleolar RNA, C/D box 46
RQ	Relative quantification
RT	Reverse transcription
Sec	Second
SMC	Smooth muscle cell
TSA	Tyramide-based amplification
Tropo	Troponin
UP	Ultra-pure
UTR	Untranslated region
vWF	Von Willenbrand Factor

Abstract

Background: Heart Transplantation (HTX) is the only curative treatment available for patients with end-stage heart failure (HF).During the first year post-transplantation more than 25% of patients will go through rejection episodes and will face the risk of developing rejection with consequent graft dysfunction with an increased morbidity and mortality. Preventing and treating acute rejection is the most central task for clinicians working with transplanted patients. The ISHLT 2005 and 2013 working formulations defined the histopathologic profile of three types of rejection: Cellular (ACR) Humoral (AMR) and Mixed (MIX). Nowadays serial endomyocardial biopsies (EMB) at decreasing intervals during the first year after transplantation and laboratory tests, such as Donor Specific Antibody (DSA) measurements, remain the gold-standard in diagnosing and monitoring acute rejection but they are morbid and prone to artefacts of sampling, interpretation and testing methodologies. Therefore this histopathological assessment needs integrative new biomarkers to characterize risk stratification for outcomes in heart transplantation. To date, the exact mechanisms involved in rejection after solid transplantation are not completely understood, so investigating process that contribute to acute allograft rejection and find effective biomarkers to diagnose, monitoring and predicting rejection will be of great value for the development of improved anti-rejection strategies.

The advent of sequencing technology such as Next Generation Sequencing (NGS) is changing medical genomics by accelerating new disease biomarkers discovery.

MicroRNAs (miRNAs) are small non-coding RNA molecules (19-24 nucleotides), highly conserved, which regulate genes expression at the post transcriptional level.

Aim: The aim of this study is to identify MicroRNA (miRNAs) expression profile in the first year after heart transplantation (HTX) with Next Generation Sequencing (NGS) technology in formalin fixed paraffin-embedded (FFPE) endomyocardial Biopsies (EMBs), to characterize the three different types of allograft rejection classified as Cellular, Humoral and Mixed.

Methods: Two groups of pts. were included: a study group of 19 pts. and a validation group of 14 pts. For each patient we selected the the first formalin fixed paraffin-embedded (FFPE) monitoring endomyocardial biopsies (EMBs) positive for each types of rejection. We excluded presensitized patients (pts) with previous implantation of Left Ventricular Assistance Device (LVAD) and with previous infections. EMBs were examined for the presence of rejection according to updated international classification criteria (ISHLT 2005 and 2013).The EMBs were classified in four groups: Acute Cellular Rejection (ACR) with 12 pts ACR: >=2R, pAMR:0, DSA: Neg ; Mixed with 6 pts ACR: >=2R, pAMR>1 (i+), DSA: Pos; Antibody Mediated Rejection (AMR) with 5 pts ACR: 0, pAMR>1 (i+), DSA: Pos; Control with 10 pts : ACR:0, pAMR:0, DSA: Neg. Small RNA fraction from the study group was sequenced with NGS Ion Proton in order to define the expression of mature miRNAs. We performed subsequent analysis with edgeR package comparing in pairs the groups to identify differentially expressed miRNAs in the different rejections. We selected 13 microRNAs according to bionformatic analysis as possible biomarckers and they have been confirmed by qRT-PCR in all the pts. With multivariate logistic regression analysis we created unique miRNA signatures as

predictive model of each rejection. Moreover in situ PCR was carried out on the same EMBs to detect miRNAs expression and localization in cell types within the EMBs.

Results: The identification of the best method of extraction for short non coding RNAs in FFPE EMBs was the first result I achieved. I tested different methods *in house* and commercial available kits and I modified the protocols to obtain good quality and adeguate quantity of RNA from FFPE tissue of small EMBs for the downstream application. With NGS we obtained and analysed more than 2257 mature microRNAs in all the biopsies of the study group. The three types of rejection and control groups were compared in pair with the un-supervised analysis showing a typical profile for each group of differentially expressed miRNAs; in particular: Mixed vs AMR: only 2 miRNAs overexpressed in the Mixed group suggesting a similarity between the two types. ACR vs AMR: 18 miRNAs overexpressed and 2 miRNAs under-expressed in the ACR. Mixed vs ACR : 7 miRNAs underexpressed and 39 miRNAs over-expressed in the ACR group. The analysis revealed that there are de-regulated microRNAs between the three rejections confirming our hypothesis that microRNAs can characterize the three pathological conditions. MiRNAs have been selected for further evaluation and validation, based on the number of reads resulting by NGS, on their highly significant FDR (< 0.05) or fold change, p-value and their involvement in relevant processes related to rejection as shown by a bioinformatic analysis based on validated target genes and reported in public databases such as TarBase (version 6.0) (111) , miRTarBase (112) , miRWalk (113), miRecords (114), DIANA-microT-CDS (115) , miRmap (116), miRDB (117) , TargetScan (118), and miRanda (119). At the end we selected 13 microRNAs. To

validate the NGS data through qRT-PCR we enrolled other EMBs from 14 pts selected according to our criteria and we tested on all the 33 EMbs, both the study and validation cohort, the selected microRNAs.

The validation analysis has shown a similar expression pattern for all microRNAs in particular: 6 hsa-miRNAs: 29c-3p/-29b-3p/199a-3p/190a-5p/27b-3p/302b-3p can differentiate all rejections compared to controls; 3 hsa-miRNAs: 31-5p/144-3p/218-5p are peculiar of AMR and MIX compared to control and ACR 2 hsa-miRNAs: 451a/208a-5p identify MIX compared to controls. Using miRNAs expression as co-variate and disease status as dependent variable we created logistic regression models: MIX:(miR-208a ,126-5p, 135a-5p); ACR:(miR-27b-3p, 29b-3p,199a-3p, 208a, 302b-3p); AMR: (miR-208a, 29b-3p, 135a-5p, 144-3p) identifying with high specificity and sensitivity each types of rejection. Finally with in situ PCR we detected some of these microRNAs in different cell types: miR-29b-3p was mostly expressed in smooth muscle cells in ACR; miR-144-3p was expressed in macrophages and in endothelial cells; moreover the expression of this microRNA in macrophages was predominant and diffuse in the ACR compared to AMR. miR-126-5p was expressed in ACR and AMR samples not only in in endothelial cells but also in Cardiomyocytes and smooth muscle cells. For MicroRNA 451a we found a co-localization of signal in endothelial cells and in lymphocytes.

Conclusions: This study demonstrate that MicroRNAs can be obtained easily from FFPE tissues, miRNAs differentially expressed are involved in pathophysiological mechanisms of rejection such as immune system cells cycle regulation and proliferation, , inflammatory pathways NFkB mediated and endothelial remodelling. According to our results the miRNAs up or down expressed modulate these

pathways in a way peculiar for the different type of rejection. The regressive models might represent a powerful diagnostic tool and in situ detection of the miRNAs casts new light on the pathophysiological mechanisms of rejection. Moreover the expression of MiRNAs 144-3p, 126-5p, 29b-3p and 451a identified by in situ PCR in endothelial cells, smooth muscle and inflammatory cells are diagnostic and are potential pharmacological targets for rejections.

Sommario

1.Introduction

1.1.1 MicroRNA biogenesis and mechanisms of action

The first identified miR, lin-4, was first described in Caenorhabditis elegans over two decades ago. This miR was found to bind to the 3' untranslated region (UTR) of the gene lin-14, suppressing gene expression (1). In 2001, paralleling the annotation of the human genome, several publications detailed the use of newer genomic technologies to annotate hundreds of mammalian miRs (2, 3). With application of next-generation sequencing technologies to discover small RNA molecules, the number of known miRs has grown exponentially in the past decade to over 2000 annotated miRs in Homo sapiens (4) (Fig.1.1).

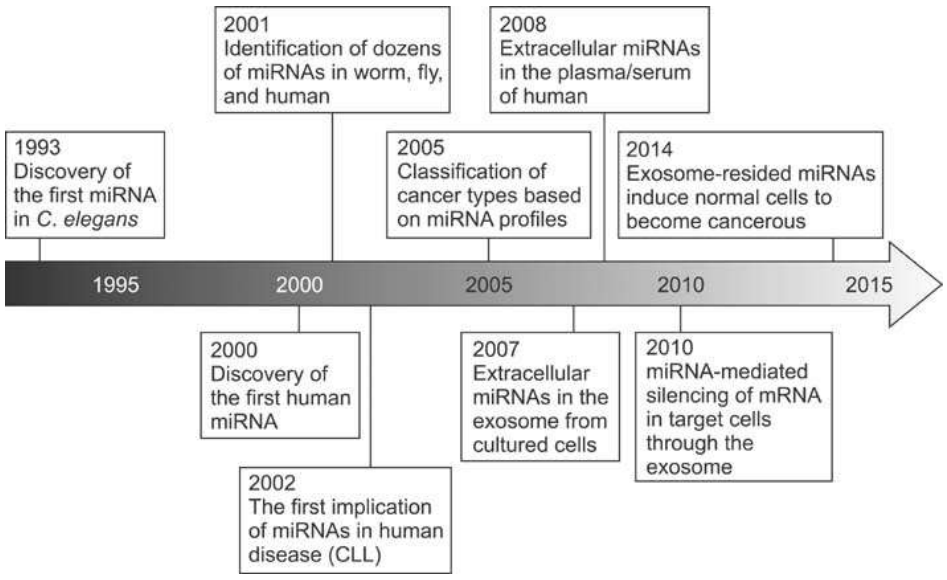


***Figure 1.1 Timeline of miRNA discovery**. Kym YK, 2015*

The importance of miRs in gene regulation is highlighted by their evolutionary conservation across mammalian species. Approximately 55% of miRs found in C.

elegans are conserved in humans (5). MicroRNAs are found in both the intergenic and coding regions of the genome. Within genes, miRs are transcribed from both intronic and exonic regions by RNA polymerase II and are often co-transcribed with the protein-coding genes within which they are contained (Fig.1.2) . Approximately, 50% of all protein-coding genes are under the control of miRs. Often, a single mRNA may be regulated by multiple miRNAs; conversely, a single miRNA may regulate multiple mRNAs (6). Complementary binding of miRs to mRNAs is regulated primarily by the "seed region" which is constituted by miR nucleotide positions 2 -7.

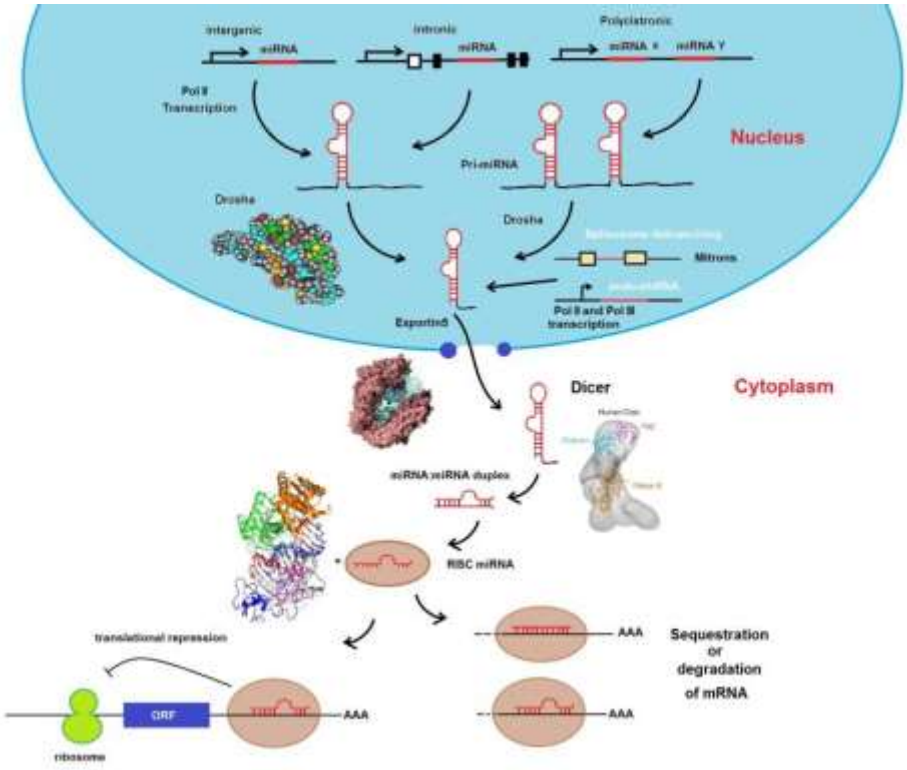


Figure 1.2. mirRNAs Biogenesis. *Production of miRNAs starts in the nucleus with the polimerization of the primary hairpin miRNA transcript (pri-miRNA) by RNA polymerase II or III, followed by the cleavage and digestion of the pri-miRNA by the microprocessor complex (Drosha–DGCR8). The resulting transcript is the pre-miRNA, which is exported to the cytoplasm by Exportin-5–Ran-GTP. Once in the cytoplasm, Dicer, TRBP and Paz proteins cleave the pre-miRNA hairpin and digest it to produce a mature duplex miRNA. Then, one of the strands is loaded onto the RISC complex and finally this guides the miRNA to its mRNA target to silence it by direct degradation or by translational repression*

The main characteristic of animal miRNAs is that their genes are often closely clustered on the genome, and in many cases are processed from the same polycistronic precursor transcript. When clustered miRNAs have similar sequences and they usually contribute additively to the regulation of a set of mRNA targets; on the other hand, when clusters contain miRNAs of distinct sequences, they are co-ordinately deployed towards their various targets (7). The genes encoding for miRNAs are present in our genome and contain the TATA box necessary for the binding of the RNA polymerase II (Pol II) responsible of their transcription; however, the possibility that a few miRNA genes might be transcribed by other types of RNA polymerases cannot be completely excluded. Transcription of miRNA genes generates long primary transcripts (pri-miRNAs) that contain a stem-loop structure which is cleaved to release the precursor of miRNA (pre-miRNA, about 70 nucleotides). This cleavage is performed by the nuclear RNase III Drosha, a large protein that requires a cofactor, DGCR8 (or Pasha), a protein which contains two double-stranded RNA-binding domains (dsRBDs) necessary to assist Drosha in substrate recognition. After that, pri-miRNA is exported to the cytoplasm by exportin-5 where is processed into 22-nt miRNA duplexes by the cytoplasmic RNase III Dicer. Mature miRNAs are incorporated into the effector complexes, which are known as 'miRISC' (miRNA-containing RNA induced silencing complex) (8). During RISC assembly, miRNA duplexes are rapidly converted into single strand, which is the mature miRNA; one strand is called -5p, whereas the second strand of this short-lived duplex disappears or it is later incorporated in RISC and called miRNA-3p. The mature miRNA that binds to complementary sequences in the 3' UTR of target mRNA causes the silencing of the gene. The effect of this binding

depends on the presence of perfect pairing or of mismatches: if the pairing it's complete, it will cause the degradation of the target mRNA, whereas a mismatch will result in translational inhibition without the reduction of the mRNA level. Because a single miRNA can target numerous mRNAs, often in combination with other miRNAs, their involvement in complex regulatory networks needs to be better understood(9).

1.1.2 MicroRNAs as diagnostic and therapeutic tools

The Biomarkers Definition Working Group (Biomarkers Definition Working Group, 2001) has defined as biomarker "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". It is possible to think to a biomarker as a trait biomarker when indicates susceptibility to a disease, a state biomarker if it defines the diagnostic of a disease, or a rate biomarker, when it tracks progression of the disease (10). It means that a biomarker is represented by a biochemical or molecular element expressed during a physiological or pathological condition in a specific stage of life, that can be used to diagnose, prognosticate and monitor the disease itself before and/or during the therapy. The identification of specific biomarkers at early stages of the disease could lead to early drug treatment, delaying the progression of the disease, and monitoring the treatment. Moreover a biomarker should meet requirements such as easy accessibility, sufficiently high specificity and sensitivity, low costs and standard applicability in the laboratories. MicroRNAs are strong and specific gene regulators and therefore promising

candidates to be diagnostic biomarkers and as well potential therapeutic targets. MiRNAs are highly abundant, tissue-specific (some miRNAs are specific to tissues or to biological stages), quantifiable, stable, with no post processing modifications. In the laboratory, the use of miRNAs as biomarkers could be advantageous, as they are stable in different extreme conditions, like for example high temperatures, high or low pH, extended storage, freeze-thaw cycles, and resistant to degradation by RNase-rich blood environment (11).

Many studies showed that the miRNAs profile is altered in patients affected by various diseases compared with that of healthy subjects (12) (13); these discoveries have attracted much interest in utilizing the miRNAs as novel biomarkers. Although their initial discoveries were made in Caenorhabditis elegans (1), the detection of miRNA homologs in many vertebrate species was what really triggered intense research efforts to start unveiling basic concepts of miRNA biogenesis and function. Today, thanks to many profiling and gain-and-loss of function studies, it has become widely appreciated that miRNAs play a major role in many different diseases in both animals and humans. (14) As a matter of fact, microRNAs possess most of the characteristics of the ideal biomarker, considering analytical criteria and clinical utility. They are specific to the pathology of interest, a reliable indication of the disease before clinical symptoms appear and sensitive to physiological or pathological changes. Compared to currently used biomarkers, which are generally based on the levels of specific proteins in the blood, biomarkers based on miRNA levels have, in theory, some advantages: they can be measured rapidly and accurately using high throughput sequencing technology but, even more importantly from a diagnostic standpoint, since miRNAs are expressed differentially in different tissues

and cellular states, the combination of a panel of miRNAs could provide a wealth of information.

First demonstrations of the ability of microRNA expression patterns to be classifiers came in the first decade of 2000. Lu and colleagues implemented a bead-based microRNA profiling method in order to assess microRNA expression in normal and tumor tissues. Unexpectedly, they observed that precise pattern of microRNAs expression can, not only distinguish tumor origin, but also the degree of differentiation and classify poorly undifferentiated tumor tissues (15). Other evidences came from microRNA signatures that could discriminate between lung tumor tissues and correspondent non tumour tissues. Differential expression was also seen between adenocarcinoma (AD) and squamous cell carcinoma (SCC) tissues and between distinct prognosis (16). A wider analysis on 22 different types of tumor tissue, revealed a signature of 48 microRNAs able to reach a classification accuracy >90% (17) . It is important to mention how NGS (Next Generation Sequencing) technologies revolutionized this field becoming progressively fundamental tools for personalized medicine (18).

Pattern of microRNAs expression may be used to classify subpopulation of patients in order to choose the right strategy in the clinical practice. However, we have to be aware that the

microRNA signatures as biomarkers are not always due to a direct biological mechanism, but also to indirect specific consequence of the disease. Biomarkers can stratify patients upon different aims. One of the first clinical questions could be to understand whether the physician is facing a pathological condition. Therefore, discriminating between tumor tissues and non-tumor tissues is extremely important.

The most recurrent example is miR- 21 which is over-expressed in many cancer types (19) (20) (21) (22) (23) The problem of using such microRNA as a biomarker is the absence of specificity. Therefore, signatures of a pattern of microRNAs are generally preferred to deliver a specific diagnosis. A nine microRNAs signature was able to discriminate between breast cancer tissues and normal cancer tissues collected by TCGA, with a high accuracy value and AUC of 0.995 (24). Another example comes from the He group which found five microRNAs (miR-424, miR-326, miR-511, miR-125b-2 and miR-451) able to provide high diagnostic accuracy of hepatocellular carcinoma starting from microRNA expression profiles of 377 hepatocellular carcinoma patients (25). As finding the pathological condition is relevant, the step forward is to understand what type of condition the clinician is facing. It is well-known that each cancer type is composed of several subtypes coming from different cellular origins and each of them has to be treated accordingly.

On the other hand, genetic models of specific miRNAs, in combination with the ease by which miRNAs can be therapeutically manipulated in vivo, have generated compelling efficacy data and motivated the biotechnology community to start exploring some of these miRNAs as drug targets in a diversity of diseases. Safety is an important determinant in drug development, so the miRNAs that are more tissue restricted will be preferred, especially for the more chronic indications. Additionally, because drug development is guided by the financial gain, another consideration will be the market for the disease indication in which the miRNA therapeutic would be applicable and how the miRNA therapeutic would compare to the current standard of care from an efficacy and financial standpoint.

One way to therapeutically mimic or re-express a miRNA is by using synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest, with modifications for stability and cellular uptake. The “guide strand” is identical to the miRNA of interest, whereas the “passenger strand” is modified and typically linked to a molecule such as cholesterol for enhanced cellular uptake. However, it should be noted that although this method would replace the miRNA levels lost during disease progression, it will also result in the uptake by tissues that do not normally express the miRNA of interest, resulting in potential off target effects. Delivery to the appropriate cell type or tissue is an important aspect of effective miRNA mimicry to prevent unwanted side-effects. Another way to increase the level of a miRNA is by the use of adenoassociated viruses (AAV). Delivered in viral vectors, the miRNA of interest can be continually expressed, resulting in robust replacement expression of miRNAs downregulated during disease. Additionally, the availability of a number of different AAV serotypes allows for the potential tissuespecificity due to the natural tropism toward different organs of each individual AAV serotype as well as the different cellular receptors with which each AAV serotype interacts.The use of tissue-specific promoters for expression allows for further specificity in addition to the AAV serotype. Furthermore, AAV is currently in use in a number of clinical trials for gene therapy, of which the safety profiles have looked quite well. In line with this, Kota et al (26) recently showed AAV-mediated delivery of miR-26a blunts tumor genesis in a mouse model of liver cancer. However, by far the most widely used approach to regulate miRNA levels in vivo is by using anti-miRs. Anti-miRs are modified antisense oligonucleotides harboring the full or partial complementary reverse sequence of a mature miRNA that can reduce the endogenous levels of an

miRNA. The key requirements for an anti-miR are that the chemistry must be cell permeable, cannot be rapidly excreted, must be stable in vivo, and should bind to the miRNA of interest with high specificity and affinity (27) (28). Several modifications have been used in vivo thus far. These chemical modifications include 2'-O-methyl-group (OMe)-modified oligonucleotides and locked nucleic acid (LNA)-modified oligonucleotides, in which the 2'-O-oxygen is bridged to the 4'-position through a methylene linker to form a rigid bicycle, locked into a C3'-endo (RNA) sugar conformation (29). Another chemical modification applied to enhance oligonucleotide stability is the balance between phosphodiester and phosphorothioate linkages between the nucleotides, with phosphorothioate providing more stability to the oligonucleotide and making it more resistant to nucleases. Nowadays anti-miR therapeutics are currently more advanced than the miRNA mimics, and many are actively pursued as clinical candidates (Fig 1.3) (30). Clinical trials are currently underway for a miR-122 inhibitor to treat hepatitis C virus (HCV) (31) , a miR-21 inhibitor to treat Alport nephropathy (https://clinicaltrials. gov/ct2/show/NCT02855268?term=rg-012&rank=1) and a miR-34a mimic to treat liver cancer (32) .

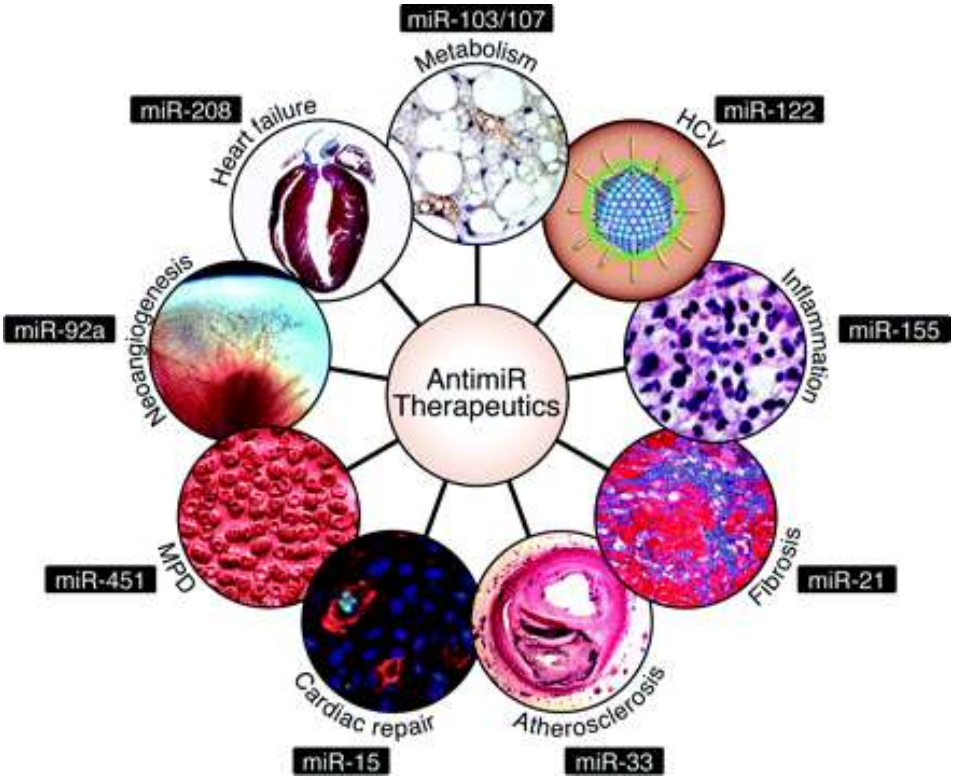


Figure 1.3. Specific miRNAs that are currently being pursued as clinical candidates. *A subset of the miRNAs of which inhibition has shown therapeutic promise and that are currently actively being pursued as clinical candidates for various disease indications.*

1.1.3 MicroRNAs in Cardiovascular Pathology

The homoeostasis of the vascular system depends on the functionality of endothelial cells and coordinated regulation of angiogenesis, vasculogenesis, and vessel regression. Cardiovascular disease is the predominant cause of human morbidity and mortality in developed countries. As such, extraordinary effort has been devoted to determining the molecular and pathophysiological characteristics of the diseased heart and vasculature with the goal of developing innovative diagnostic and therapeutic strategies to combat cardiovascular disease. The collective work of

multiple research groups has uncovered a complex transcriptional and post-transcriptional regulatory circuit, the integrity of which is essential for maintenance of cardiac homeostasis. Mutations in or aberrant expression of various transcriptional and post-transcriptional regulators have now been correlated with human cardiac disease and pharmacological modulation of the activity of these target genes is a major focus of ongoing research. MicroRNAs have been implicated in virtually every cardiovascular disorder in which they have been examined, including heart failure, cardiac hypertrophy, remodeling after myocardial infarction, arrhythmias, atherosclerosis, atrial fibrillation, and peripheral artery disease. Virtually all of the basic cellular processes involved in cardiovascular development and disease, such as cell proliferation, differentiation, apoptosis, fibrosis, angiogenesis, and inflammation are subject to miRNA control (Fig 1.3).

In Heart Failure (HF), the role of miRs as potential biomarkers is developing rapidly. One of the earliest miR biomarker studies in HF investigated the ability of 16 miRs, using a microarray hybridization platform, to distinguish the etiology of dyspnea in patients presenting to the emergency department. Tijsen and colleagues determined that miR 423-5p had a strong ability to diagnose HF (area under the curve, AUC of 0.91), but the miRs correlated weakly with other HF measures such as left ventricular ejection fraction or natriuretic peptide levels (R = 0.43) (33). Expanding on this initial work, Goren and colleagues isolated miRs from serum exosomes and used polymerase chain reaction (PCR) to quantitate 186 miRs in 30 patients with HF and 30 controls. Although miR-423-5p had a good diagnostic accuracy for HF (AUC of 0.88), the investigators created a 4 miR-panel composed of

miR-22, miR-92b, miR-320a, and miR-423-5p, which modestly improved the discriminatory power to an AUC of 0.90. The Spearman correlation for the miR-panel and BNP was 0.63, indicating only a moderate correlation (34). In addition to diagnosing HF, low levels of miR-423-5p and miR-30d have been shown to portend a poor prognosis in HF, suggesting that these miRs may have specific cardioprotective properties (36,37).

Myocardial remodeling is typically characterized by cardiomyocyte (CMC) hypertrophy, CMC

apoptosis, interstitial fibrosis, and aberrant cardiac conduction, which ultimately impair the

electrico-mechanical performance of the myocardium. Pathological hypertrophy is a maladaptive process that ultimately leads to reduced cardiac output and is an independent risk factor in heart failure (35). Pathological cardiac hypertrophy occurs primarily upon pressure overload due to arterial hypertension or stenosis of the aortic valve, as well as inherited mutations in sarcomeric and cytoskeletal proteins. Several individual miRNAs are transcriptionally regulated during cardiac hypertrophy and heart failure. Some of these have been experimentally verified to play important roles in cardiac development and disease:

miR-1—miR-1 is encoded by two genes (miR-1-1 and miR-1-2), each of which is co-expressed bicistronically with one copy of the two miR-133a genes. miR-1 expression is restricted to heart and skeletal muscle and regulated by the transcription factors SRF and MEF2/MyoD, respectively (36). During cardiogenesis, miR-1 is

believed to control the balance between differentiation and proliferation of cardiac precursor cells by targeting Hand2, a transcription

factor that promotes expansion of ventricular CMCs. Besides the crucial role of miR-1 during cardiac development, it is currently unclear whether miR-1 contributes to adverse remodeling in human heart failure. Some studies suggest upregulation of miR-1 expression in heart failure (37), while others report downregulation (38).

miR-21—miR-21 is the most upregulated miRNA in cardiac disease, although under basal conditions is only weakly expressed (Figure 1). miR-21 is also highly upregulated in several cancers and believed to function as an oncogene by inhibiting apoptosis (39). The role of miR-21 in cardiac disease is controversial at present. Some studies find an induction of CMC hypertrophy by miR-21 in vitro (40) and indirectly in vivo via fibroblasts (41). In contrast, other studies report an antihypertrophic effect of miR-21 in isolated cardiomyocytes; The reasons for the discrepancy between these studies are unclear, however miR-21 is predominantly expressed in cardiac fibroblasts, not CMCs

miR-23—There are two miR-23 genes that differ by only one nucleotide in the mature miRNA sequence. Each miR-23 gene is closely clustered with a miR-24 and a miR-27 gene, suggesting that they are transcribed as a common transcript. Accordingly, several groups found that miR-23, miR-24 and miR-27 are all upregulated in heart failure and murine cardiac hypertrophy

miR-133—The miR-133 family contains three miRNA genes: miR-133a-1, miR-133a-2, and

miR-133b, which are each transcribed as bicistronic transcripts together with miR-1-2,

miR-1-1, and miR-206, respectively. Knockdown of miR-133 in mice with specific antagomiRs was shown to induce cardiac hypertrophy, suggesting that pharmacological elevation of miR-133 expression might prevent cardiac hypertrophy during cardiac disease (42)

MiR-208a is a cardiac-specific miRNA that is generated from the intronic region of the gene that codes for a major protein that regulates myocardial contractility in the adult heart, α-myosin heavy chain (α-MHC) (43) . MiR-208a has been shown to regulate critical cardiac transcription factors and is required for proper cardiac function. Specifically, overexpression of miR-208a resulted in increased α-MHC expression and was associated with arrhythmias, fibrosis and hypertrophy in mice (44).

Cardiac conduction: Membrane excitability is a special characteristic of CMCs and is regulated via ion channels. Specifically, Na+, Ca2+, and K+- channels and gap junction proteins such as connexin 43 are important regulators of CMC polarization and depolarization during contraction and relaxation, respectively. Several miRNAs, including miR-1 and miR-133, are predicted to target ion channels and might therefore play important roles in cardiac conduction and the onset of arrhythmias during cardiac disease.

miR-1-2 knockout mice showed several ECG alterations such as reduced heart rate, shortened PR-interval and widened QRS complexes and died due to cardiac arrhythmia. The ECG alterations were presumably at least partially due to elevated expression of the miR-1 target Irx5, a transcription factor that regulates the

expression of Kcnd2, a potassium channel important for normal cardiac repolarization (45)

Cardiomyocyte apoptosis and regeneration: Since the adult heart has only limited regenerative capacities, an excessive loss of CMCs following myocardial ischemia or infarction can significantly decrease cardiac performance. Some miRNAs seem to play important roles in the regulation of CMC apoptosis in vivo.

miR-199a—The miR-199 family contains 3 miRs: miR-199a-1, miR-199a-2 and miR-199b that are all encoded by the antisense strand of an intron of a dynamin gene (Dnm2, Dnm3 and Dnm1, respectively) Downregulation of miR-199a de-repressed the expression of hypoxia-inducible factor (Hif)-1α, the most important transcription factor for induction of gene expression upon hypoxia. miR-199a downregulation also resulted in the de-repression of Sirtuin 1, which was responsible for downregulation of prolyl hydroxylase (PHD) 2, the enzyme that hydroxylates Hif-1α to induce its degradation (46).

Fibrosis: Fibroblast activation and proliferation during cardiac disease leads to inappropriate secretion of extracellular matrix proteins and concomitant interstitial fibrosis. Fibrosis results in impaired cardiac contractility and alters the electromechanical characteristics of the myocardium, often leading to arrhythmias, an important cause of mortality in heart disease**.**

The expression of several miRNAs is altered following myocardial infarction (MI) or other fibrotic pathologies.

miR-133a double knockout mice develop severe fibrosis and heart failure (47). Downregulation of miR-133 during cardiac disease might therefore result in increased expression and secretion of CTGF from CMCs, which consecutively stimulates extracellular matrix synthesis in fibroblasts.

miR-21: The fibroblast-enriched miR-21 is upregulated in failing and hypertrophic myocardium, possibly as a consequence of fibroblast proliferation. Thum et al. (2008) demonstrated that miR-21 increases fibroblast survival and fibrosis possibly via inhibition of sprouty homologue 1 and consecutive ERK-MAP kinase activation.

miR-29: van Rooij et al. (2008) found all members of the miR-29-family downregulated after myocardial infarction, particularly in the border zone (48). MiR-29 is predicted to target myriad genes that are involved in fibrosis such as collagens, fibrillins, and elastin, and is a prime example of the ability to modulate a large portion of a particular pathology by pharmacologically targeting one miRNA.

1.1.4 MicroRNAs in Vascular System and Vascular Diseases

Vessel injury is characterized by profound phenotypic changes in molecular and physiological identity; vascular smooth muscle cells (VSMCs) in particular undergo a program of dedifferentiation and become more proliferative and migratory after injury. These changes contribute to neointimal thickening during proliferative vascular diseases such as atherosclerosis, hypertension, and restenosis.

Several groups have demonstrated roles for various miRNAs in SMC phenotypic modulation and the response of the vasculature to injury.

miR-21: The results of Ji et al. (2007) demonstrated that miR-21 promoted SMC proliferation following vessel injury via inhibition of PTEN and the subsequent

activation of the PI3K/Akt signaling pathway, which is partially blocked upon knockdown of miR-21 (49).

miR-221—miR-221, although not VSMC-specific, is induced in VSMCs upon plateletderived growth factor (PDGF) stimulation (50). Activation of the PDGF signaling pathway results in the switch of VSMCs from a fully differentiated, contractile state to a less differentiated, synthetic state typified by increases in proliferation and cell migration, contributing to the formation of a neointimal lesion following arterial injury

Angiogenesis: Neoangiogenesis plays an essential role in the process of cardiac repair following ischemic

injuries such as myocardial infarction (MI) by promoting vascularization of the infarcted tissue through growth of collateral vessels that bypass the infarcted artery. Various growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are required for proper generation of blood vessels after MI.

miR-221 and -222—Studies using human umbilical vein endothelial cells (HUVECs) migration,

and endothelial tube formation demonstrated miR-221 and -222 regulate angiogenesis in response to Stem Cell Factor (SCF), migration, and endothelial tube formation (51).

miR-126—miR-126 has been implicated in the maintenance of vascular integrity and promotion of vessel growth as a pro-angiogenic factor both in vitro and in vivo (52)

(53) (54). The pro-angiogenic effect of miR-126 was attributed, at least in part, to the repression of Spred-1, an intracellular inhibitor of VEGF and FGF-mediated angiogenesis and phosphotidylinositol-3-kinase regulatory subunit PIK3R2 (p85β)

1.1.5 MicroRNA in solid organ transplantation

Rejection is one of the major causes of allograft failure and preventing and treating acute rejection are the central task for clinicians working with transplant patients. Critical advances have been made in the transplant field during the last decades. Most of this progress relates to short term outcomes as consequence of improved surgical techniques and the use of new and more powerful immunosuppressive therapies. Unfortunately, the progress achieved in short term outcomes does not translate to long term outcomes. This may be partially consequence of the lack of a robust gold standard to monitor graft function (55). Appropriate immunosuppression represents a challenging situation, including a delicate balance between rejection rates and chronic allograft dysfunction as well as immunological and non-immunological side effects (56, 57). Surprisingly, graft monitoring has not changed significantly since solid organ transplantation was pioneered. Post-transplant monitoring still relies on the surveillance of allograft function and an acute alteration in this functional parameter usually requires an invasive allograft biopsy (58). Allograft biopsy represents the gold standard for diagnosis of conditions like acute rejection (AR), disease recurrence and drug toxicity (59, 60). However, allograft biopsy often relies on “subjective” measures, with high variability in results and reporting methods among pathologists or limited diagnostic accuracy associated with sampling error (59). Therefore is evident that there is a critical need for biomarkers for early diagnosis, treatment response, and outcome prediction in organ

transplantation, with the final goal of predicting the individual's risk of allograft injury, leading to an individualized treatment. Advances in understanding the molecular basis of disease using genomics and proteomics technologies have provided new opportunities to develop genomic-based tools to diagnose, predict disease onset or recurrence, tailor treatment options, and assess treatment response (61) (62) (62). However, there is still no routine application of any of these markers in clinical transplantation. Novel and easily accessible biomarkers of acute rejection could make it possible to detect rejection earlier and make more fine-tuned calibration of immunosuppressive or new target treatment possible. Studies to identify non-invasive biomarkers of rejection and its underlying molecular events have increased significantly during the last years (61) (62), but new accurate markers are still lacking. Accumulating evidence underlines a critical function for miRNAs in the modulation of innate and adaptive immune responses (63) (64) (65). MiRNAs have the potential of being reliable biomarkers because they are tissue specific, stable in different biological fluidics (including archival samples), relate with clinical conditions, and can be measured using cost-effective technology. In addition, further discovery of the association between miRNAs and diseases would provide potential targets for novel therapy in transplantation. Evaluation of tissue graft and/or circulating miRNA profiles may accelerate the use of new biomarkers in guiding the diagnostic, therapeutic, and prognostic strategies that associates with over-immunosuppression, organ toxicity, and graft rejection or loss. The publications that exist on the topic of miRNAs and transplants focus on miRNAs isolated from biopsies. (66) Now we focused on the evaluation of miRNAs as potential biomarkers in different solid organ transplantation:

Kidney: The implication of miRNAs in solid organ transplantation has been most extensively studied in renal transplantation and in the context of renal allograft rejection, miRNA expression profiles have been investigated in biopsy samples, peripheral blood and urine samples.

Renal Biopsies: Sui et al. studied miRNAs in renal allograft biopsies from three patients with acute T-cell mediated rejection (TCMR) and three normal kidney cortex specimens They reported that the expression pattern of 20 miRNAs (12 down-regulated and 8 up-regulated) distinguished acute rejection biopsies from normal renal cortex (67) one other study that measured the expression of miRNAs in sixty-five FFPE renal allograft biopsies from patients

undergoing TCMR, antibody-mediated rejection (ABMR) and delayed graft function (DGF) and compared the expression profile to that of protocol biopsies (68). Unsupervised hierarchical clustering of the 65 regulated miRNAs identified distinct miRNA signatures. Patients developing rejection (30 samples for cellular rejection and 11 samples for humoral rejection) were clearly separated from the control group (10 samples). Interestingly, six miRNAs identified in this work (miR- 155, miR-125a, miR-30c, miR-27b, miR-193b, and miR-125b) were previously identified in other study using different types of samples and different miRNA profiling platforms (69).

Blood: Danger et al. studied the miRNA expression pattern in peripheral blood associated with chronic antibody mediated rejection (CAMR) of the renal allograft.

They observed an increase in miR-142-5p expression in PBMCs of patients with CAMR compared to patients with stable graft function. An ROC curve analysis performed on independent samples showed that the miR-142-5p expression level in PBMCs could distinguish CAMR from stables subjects with a good diagnostic performance. (70)

Urine sample: The development of detectable biomarkers in urine is naturally appealing to nephrologists not only because it provides a strictly non invasive test but also because the urine specimen provides a representative sampling of the entire kidney allograft .

Lorenzen et al. studied the urinary miRNA expression profile in patients with either stable function or TCMR [44]. After demonstrating the stability of miRNAs in urine over time, they showed that miR-10a, miR-10b and miR-210 were strongly deregulated in urine samples collected during TCMR. A validation cohort confirmed that acute rejection was associated with a low urinary miR-210 expression.

Liver: A study by Farid et al. (71) investigated the potential of serum miRNAs to be used as biomarkers in liver transplantation. They studied the expression of three miRs in serum samples from liver transplant recipients and healthy controls and compared this to their expression in liver graft biopsies. They showed that the serum levels of miR-122, miR-148a and miR-194 were significantly elevated in patients with liver injury immediately after liver transplantation. Importantly, circulating miRNAs appeared to be more sensitive than aminotransferase in detecting liver injury. Similarly, acute rejection of the liver allograft was

associated with an early increase in circulating miRNAs. In the context of acute rejection, miRNAs appeared to be very sensitive, as their level increased earlier than aminotransferase levels. These results suggest that miRNAs are released into the circulation early after liver injury, promoting their use as biomarkers in liver transplantation.

Heart: Many studies have reported the use of miRNAs as biomarkers for cardiovascular diseases; however, only a few studies have investigated miRNA expression after heart transplantation, and most studies were performed in rat transplantation models. In cardiac allograft transplantation, miRs involved with rejection were first described by Wei et al. [54]. In a mouse model of rejection, the group identified both myocardial tissue- and lymphocyte-specific miRs that were differentially expressed in the rejecting allograft (72) . They then identified miR-182 as a marker of allograft rejection that was expressed at the time of rejection not only in the myocardium and graft lymphocytes, but was also found in the plasma, making it a non-invasive biomarker of allograft rejection. It appears that miR-182 may be secreted by CD4+ T cells during rejection and is in part responsible for triggering the allo-immune response (73). A separate group of investigators identified miR-155 as the most upregulated transcript in mice and humans with cellular rejection (74).

In order to identify miR biomarkers of rejection, Duong et al. (75) investigated the role of 14 pre-selected miRs (including miRs-21, miRs-142, miRs-155, and miRs-182) in 113 human cardiac transplant recipients. They identified four miRs (miR-10a, miRs-31, miRs-92a, and miRs-155) that were differentially regulated in both the tissue and serum of patients with allograft rejection . Interestingly, these individual

miRs had an excellent ability to diagnose allograft rejection and were also able to accurately differentiate cellular and antibody-mediated rejection, a significant limitation of current genomic biomarkers.

Wang et al. studied the expression of three muscle-specific miRNAs, miR-133a, miR-133b and miR-208a, in plasma from 14 healthy controls and 7 heart transplant recipients at days 0, 1, 2, 3, 7 and 14 post-transplantation (76). They observed a maximal increase in these miRNA levels early after transplantation and found that overall dynamic changes correlated well with the serum levels of troponin I.

1.2 Heart transplantation diagnosis

Heart transplant is most important therapeutic strategy for end-stage cardiomyopathies.

Pioneer of heart transplant was Christian Barnard in 1967. Up to now more than 90 000 heart transplant have been performed worldwide and registered by International Society of heart and Lung Transplantation (ISHLT). In the last decade between 3600-3850 heart transplant have been registered per every year. In our center at University of Padua the first heart transplant was performed in 1985 for Dilated Cardiomyopathy (77).

According to ISHLT data, cardiomyopathies that most frequently go to the transplant are non-ischemic (dilatative) cardiomyopathies, and post-infarction chronic ischemic heart disease, which together account for almost 85% of cases; valvular diseases account for 2-3% of transplants, while the remaining 10% is due to hypertrophic, restrictive cardiomyopathies, post-myocardial heart disease, congenital heart disease and more. In our transplant center "Centro Gallucci" in Padua, transplantation

indications are: 44% of chronic post-infarctatic ischemic heart disease, dilated cardiomyopathy for 34%, followed by 6.8% valve disease and arrhythmogenic cardiomyopathy 3.6%.

Until now, rejection in solid organ transplantation remains the most important challenge for the survival of these patients, and is clinically classified as hyperacute, acute and chronic rejection. Hyperacute rejection is currently a rare occurrence, manifested immediately after transplantation (defined as rejection occurring within 24-48 hours from transplantation, (78) and is the cause of immediate organ loss. This phenomenon depends on the antibody response of the recipient's immune system to the antigens present on the vascular cells of the organ. Acute rejection originates from the immune response of the lymphocytes and macrophages of the recipient to the tissue antigens of the new organ and may occur theoretically at any time after transplantation, most often after the first week and within the first 4 months. Chronic rejection is one of the major causes of mortality and loss of graft after transplantation and manifests itself as a coronaropathy of the graft in heart transplant.

To monitor the rejection, cardiotransplant patients undergo a surveillance protocol carried out by endocardial biospies. This procedure was proposed for the first time in 1975 by Caves et al and became of paramount importance to identify cellular rejection and apply most appropriate individual immunosuppressive therapy. The monitoring protocol provides that patients are subjected to biopsy a week for the first month, every two weeks for the second month, and every 6 to 8 weeks between the third and twelfth month. After the first year the frequency may decrease and become biannual or annual (79). Urgent and / or emerging biopsies are performed every time

there is clinical symptom or if the previous biopsy showed the presence of rejection (80).

Discovery of cyclosporine (calcineurin inhibitor) revolutionized the survival and follow-up of these patients. It was Barel in 1972 to discover the immunosuppressive properties of cyclosporin, a fungal metabolite extracted from tolypocladium inflatum. To date, it is the drug chosen in solid organ transplants, such as heart, liver and kidney. Immunosuppressive therapy is administered according to a precise therapeutic pattern and modulated according to the rejection as assessed on endomyocardial biopsy (EMB). According to the last ISHLT Registry the most frequent combination is: tacrolimus, Micophenolato Mofetil and steroids. The use of several drugs allows to contain the side effects of ciclosporin, in particular its nephrotoxicity, so patients receive triple immunosuppressive therapy, steroids, ciclosporin, mycophenolate mofetil. As an alternative to mycophenolate mofetil, other immunosuppressants such as aziatoprine or everolimus are used. Several studies in the 1990s showed that the lowest rates of rejection generally resolved spontaneously and almost always without treatment, demonstrating that they progress to a higher degree in subsequent biopsy in only 15-20% of cases. If the degree of Acute Cellular Rejection (ACR) rejection is above 2, patients need to be admitted and treated for 3 days with high doses of corticosteroids intravenously and an increase in immunosuppression, leading to the reduction of inflammatory processes associated with rejection. Patients with a grade of acute Antibody Mediated Rejection (AMR) higher than pAMR2 need to be hospitalized and have to undergo plasmaexchange cycles together with high intravenous (IVIg) dose of

immunoglobulins and rituximab as well as basic immunosuppressive therapy. Table (1.1).

AMR treatment strategies are based on modulation of antibody-induced damage, the elimination of alloantibodies (antibodies circulating against allografts), the downregulation of the production of alloantibodies by plasma cells, and the reduction of levels and 'activity of both native B cells and mature B cell. Immunosuppressive therapy therefore reduces the incidence of rejection, but complications of chronic immunosuppression include drug toxicity, neoplasia development, and increased risk of infection, so quality of life after 10 years is slightly worse for all side effects , such as hypertension, kidney failure, dyslipidemia, and diabetes.

Table 1.1 Immunosuppressive therapy for heart transplanted patients

Corticosteroids	powerful immunosuppressive and anti-inflammatory agents that affect the number, distribution and functions of all types of endothelial leucocytes and cells
Calcineurin Inhibitors.	Most important immunosuppressive agent in solid organ transplantation that reduce expression of IL-2.
IVIg	inhibit the specific alloantibodies HLA in vitro and in vivo, reduce the circulating levels of DSA and inhibit the B cellular production of DSA. IVIgs are often used to reduce antibody levels in patients who are sensitized before transplantation
Plasmaexchange	mechanically removes circulating antibodies
Globulins anti-lymphocytes	antibodies directed against T cells or timocytic lymphocytes(Thymoglobulin).
Micofenolato Mofetil	antiproliferative agent
Everolimus	antiproliferative agent
Rituximab	monoclonal antibody directed against surface antigen CD20 of B cells. Phosphorylation of the CD20 is normally involved in the regulation of development and differentiation of B cells. Rituximab binds to CD20 and interferes with the activation and differentiation of B cells.

The main causes of graft loss in the first years after transplantation were graft failure (34%), infections (21%) and acute rejection (22%).

1.2.1 Acute Cellular Rejection(ACR)

The cause of acute cellular rejection (ACR) is the recognition of the allograft, in which non-self antigens are attacked by the immune system of the host. Host dendritic cells adhere to the endothelial cells of the allograft, invade the wall of the graft vessels, capture foreign antigens, and present these alloantigens to host naive cells (81). According to the study by Lu et al (82), 75% of post-transplant patients in their follow-up show at least one episode of acute cellular rejection, especially mild. So the percentage of no rejection after one year is about 10-23%. The survival after the transplant with the presence of only acute cellular rejection at 1, 5 and 10 years is respectively 93%, 85% and 74%. According to the criteria of ISHLT 2004 acute cellular rejection is defined as the presence of lymphocytic infiltrate with or without myocardial injury. (Table 1.2)

Therefore, the ACR is characterized by a mononuclear inflammatory infiltrate with perivascular distribution, composed mainly of T lymphocytes, although other types of cells, including B lymphocytes, macrophages, neutrophils and occasional eosinophils, may be present in the most severe cases. When present, myocardial damage is characterized by the invasion of mononuclear cells at the perimeter of the myocytes resulting in irregular and jagged edges and distortion of the myocyte cell architecture. Hypereosinophilias and nuclear picnosis may be indicative of myocardial necrosis. (83) Morphologically, acute cellular rejection consists of a mononuclear inflammatory infiltrate that is primarily due to a T-mediated cell

response, with myocardial infiltration of T CD3 lymphocytes and macrophages, directed against cardiac allograft. (84) The ACR seems to be mediated primarily by CD4 + T cells that they can interact synergistically with the CD8 + cytotoxic T cells, which they produce interferon-gamma. (85) An increase in the number of antigen presenting cells (macrophages and dendritic cells) has also been observed depending on the severity of the rejection. Cell B infiltrate, composed of activated B lymphocytes and Natural-Killer cells, is rarely present in mild rejection, but increases in moderate rejection, suggesting their role as promoters and effectors of cellular rejection. (79)

Table 1.2. Comparison of ISHLT Classification of Acute Cellular Rejection (ACR) ISHLT of 1990 and 2004

2004		1990	
Grade 0 R	No rejection	Grade 0	No rejection
Grade 1 R Mild	Interstitial and/or Perivascular infiltrate with only one necrotic myocyte focus	Grade 1 Mild A-Focal B-Diffuse Grade 2 Moderate (Focal)	Interstitial and /or perivascular infiltrate without myocyte damage Diffuse infiltrate without myocyte damage Only one infiltrate focus with myocyte damage
Grade 2R moderate	Two or more inflammatory infiltrate foci + myocyte damage	Grade 3 Moderate A-Focal	Multifocal infiltrate with myocyte damage
Greade 3 R Severe	Diffuse infiltrate + multifocal myocyte damage	B-Diffuse	Diffuse infiltrate with myocyte damage Diffuse infiltrate of polimorphonucleate

	± edema ± haemorrhage ± vasculitis	Grade 4 Severe	cells with extended myocyte damage ± edema ± hemorrhage ± vasculitis

1.2.2 Antibody Mediated Rejection (pAMR)

The first description of AMR as a clinical entity was made by Herskowitz et al in 1987 (86) which described it as a type of rejection characterized by arterial vasculitis and with low outcome in post-transplant patients, and recognized the importance of Identify the presence of immunoglobulins and complement in the frozen sections and the type of inflammatory cells in biopsies. Hammond HE et al in 1989 (87) was the first to describe the "vascular" rejection phenomenon by detecting a correlation between myocardial damage in the absence of inflammatory cells and the presence of "graft disfunction" (14). In the new classification of cellular rejection still active today, it was termed humoral rejection for the first time with well-defined histopathological and immunological criteria. The AMR is associated with a worse survival of the graft and was observed in sensitized patients, including those who had previous transplantation, pregnancy or mechanical care equipment prior to transplantation. (83) Among the risk factors for humoral rejection, the female sex, CMV seropositivity, and the high responsiveness of reactive antibody panels (PRAs) were described. According to Gregory and Michael Fishbein's study (88) AMR tends to occur soon after transplantation, usually in the first month. However, in patients with pre-existing antibodies against the donor HLA, AMR may also be seen within the first week.

At first AMR had been described as a clinical-pathological continuum that

proceeds through 4 phases (89):

- latent humoral response: development of antibodies only;

- silent phase (accomodation): with circulating antibodies and C4d deposition, without any histological or clinical manifestations.

- Subclinical phase: with circulating antibodies, C4d deposition, histological and immunopathological alterations;

- symptomatic AMR: with circulating antibodies, C4d deposition, histological and immunopathological alterations and also clinical manifestations such as reduction of heart ejection fraction and ECG abnormalities.

The pathological features of the cardiac AMR include activation and swelling of the capillary endothelium, acute inflammatory infiltrate of macrophages and / or neutrophils, as well as deposition of immunoglobulins and complement within the vessels on the endothelium and overregulation of the antigens of the higher complex class II histocompatibility. Interstitial haemorrhage, fragmentation and capillary congestion, mixed inflammatory infiltrates, thrombosis, endothelium picnosis, and / or carotid artery and marked oedema are recognized as severe or advanced AMR characteristics. In 1990 SHLT (90) proposed as criteria for the diagnosis of AMR the characteristics of "positive immunofluorescence, vasculitis or severe oedema, in the absence of cell infiltrates". The presence of vasculitis, understood as adherence of macrophages to capillary endothelium, is considered a marker for the diagnosis of AMR, from mild rejection to severe rejection. According to the 2004 IHLT criteria for humoral rejection, AMR was omitted (AMR 0), in the absence of histological or immunopathological features of AMR, or present (AMR 1), in the presence of histological features, identification of antibodies (directed against CD68, CD31,

C4d) and presence in serum of specific antibodies (DSA), without increasing its severity (83). In 2004 the ISHLT redefined the characteristics for the diagnosis of AMR. (83) (91) Histologically, on colored slides with hematoxylin and eosin (H & E), AMR is characterized by capillary myocardial injury with swelling of endothelial cells (and nuclei enlargement) and accumulation of intravascular macrophages. In addition, interstitial edema and hemorrhage may be present along with the neutrophils inside and around the capillaries. There may also be intravascular thrombi and myocardial necrosis without cell infiltration. (see Table 1.3).

Through Immunofluorescence or immunohistochemistry can be found:

- immunoglobulin (IgG, IgM and / or IgA) with the deposition of the complement (C3d, C4d and / or C1q) in capillaries, immune fluorescence (IF) of frozen sections;
- positive intracapillary macrophages with CD68 (positive CD31 and / or CD34) immunization to immunocytochemistry (IHC);
- positive C4d coloring of capillaries to immunocytochemistry

Reed et al. in 2006 stated that the definitive diagnosis of AMR requires the presence of insufficient transplantation and / or the presence of circulating DSA (antibody-specific circulating antibodies).

Table 1.3. Histologic definitions

Endothelial swelling = swelling of cytoplasm and nuclear enlargement and / or accumulation of endothelial cells with cytoplasmic protrusions in the lume, which causes relaxation and narrowing of the vascular lumen
Activated intravascular mononuclear cells (IAMCs) = accumulation of polymorphonuclear cells, such as macrophages and leukocytes, within capillaries and veins that appear enlarged and with filled vascular lumens.
Oedema = interstitial enlargement and separation of myocytes.
Haemorrhage = red interstitial cells.
Microtrombi = intravascular thrombi with fibrin deposition or platelet aggregates.
Myocardial damage = vacuolization of cytoplasm or loss of myofilaments.
Myocardial Necrosis = coagulative or colliquative necrosis.
The definition of " Accommodation " refers to the condition in which the graft remains structurally intact and continues to function without antibody mediated damage, despite the presence of circulating DSAs and the deposition of C4d

During the second half of the year 2000 there were numerous pathophysiological and clinical studies on humoral rejection, which were able to better define its clinical behaviour.

In 2013, the International Society (ISHLT) published a new classification of humoral rejection, which is described as:

- pAMR 0 - negative for pathologic AMR: histopathological and immunopathological studies both negative;

- pAMR 1 (H +) - only histopathologic AMR: histopathological features present and negative immunopathological characteristics;

- pAMR 1 (I +) - only immunopathologic AMR: negative histopathological characteristics and positive immunopathological characteristics; ie CD68 + and / or C4d + for IHC and C4d + with or without C3d + for IF.

- pAMR 2 - patologic AMR: histopathological and immunopathological features are both present;

- pAMR 3 - severe pathologic AMR: is characterized by interstitial haemorrhage, capillary fragmentation, mixed inflammatory infiltrates, picnoses of endothelial cells, and / or karyorrhexis marked oedema and immunopathological features. These cases may be associated with profound haemodynamic insufficiency and low clinical outcome.

The same classification also clarifies the immunohistochemical criteria for the pathological diagnosis of AMR:

- the grading of C4d: 0 (<10% of the capillaries involved) = negative; 1 (10-50% of the capillaries involved) = focal positive coloration, weak in few areas; 2 (> 50% of the capillaries involved) = multifocal / diffuse positive coloration in several areas;

- intravascular distribution of CD68: 0 (<10% of the capillaries involved) = negative; 1 (> 10% of the capillaries involved) = multifocal / diffuse intravascular macrophages, sometimes also in aggregates.

Normally when biopsies are analyzed, positive C4d is considered only when it is> 50% with a diffused capillary coloration, whereas CD68 is positive when it is> 10%, regardless of whether it is focal or diffused. Thus, all types of inflammatory cells, especially T CD3 + and CD68 + macrophages, have significantly increased in pAMR + cases compared to pAMR0 cases (92).

CD68, the cell surface antigen present on the macrophages, allows identification within the vessels, and their role in the humoral activation pathway is to act as antigen presenting cells to endothelial cells, triggering the cascade of the complement with the production and deposition of C4d and C3d.

The C4d protein is an inactive fragment formed after the complement cascade activation through the alternative pathway, and covalently binds to the endothelial cells of the vessels, and is considered an indirect marker of complement activation. Its deposition on the endothelial surface of the intraparenchymal graft capillaries is an antibody-mediated rejection marker, but has been identified AMR also with negative C4d. The antibodies are typically directed against class I and class II human HLA (leukocyte antigens). Reactive antibodies against donor HLA molecules are called specific donor antibodies (DSAs). These can be preformed and already present before transplantation or are produced de novo after transplantation. (93) Circulating DSAs are not always detected in serum when diagnosing AMR. This may be due to the absorption of DSA by the allograft. Therefore, the presence of DSA alone is not diagnostic for AMR (89). The most important targets of antibody-mediated rejection are the molecules of the higher complex of histocompatibility (MHC). MHC class I molecules are on the surface of all nucleated cells, including endothelial cells, while the distribution of Class II MHC molecules is limited to the surface of lymphocytes

B, dendritic cells, endothelial microcirculation cells and may be expressed by other cells in relation to stimuli that trigger the transcription. The extreme polymorphism of the MHC class I and II molecules favors their main function which is the presentation of T cell antigen. Production of HLA antibody specific antibodies depends on exposure to events that increase this possibility, for example pregnancy, transfusion and the transplant itself. According to the study of Rose et al (85), about 66% of transplanted patients produce lymphocytotoxic anti-HLA antibodies after transplantation. Transplanted patients presenting DSAs against HLA have more T-cell CD8 cytotoxicity than T CD4 helper cells in EMB lymphocytic infiltrates compared to non-DSA versus HLA patients. Patients without DSA have less C4d positivity than patients with anti-HLA DSA (85). These antibodies are more common in transplanted patients who had rejection and their recovery seem to precede rejection episodes (89). In their study, Tible et al (94), demonstrate that the increase in the degree of pAMR is related to the increased probability of DSA positivity and the activation of endothelial cell markers with pAMR0, pAMR 1H + and pAMR 2 exhibiting 17.6%, 77.3% and 100% DSA positivity respectively. The study of Hammond et al. 2016 (95) shows that pAMR 1H +, pAMR1I + and pAMR2 have a CAV mortality risk similar to those without pAMR, while pAMR3 gives a much higher risk. Finally AMR increases the CAV incidence of 10% to one year and 36% to 5 years after transplantation. Therefore, AMR is associated with a reduced long-term survival of the graft, but also with a higher CAV incidence than acute ACR or ACR and AMR (96).

1.2.3 Mixed Rejection (MR)

Mixed rejection (MR) is defined as the presence of ACR cellular infiltrates and histopathological and immunopathological features of AMR in EMB (90) Mixed rejection is a new entity, that has been already histologically described by Hammond HE et al in 1989 (87), but to date, not clearly studied in terms of survival and in physiopathology. MR is described as a form of rejection which includes both histological features of cellular and humoral rejection or as the presence of humoral rejection markers in the biopsy preceding cellular rejection. In this study they also showed that survival at 3 years after transplantation was 57% for AMR, 89% for mixed rejection and 95% for ACR. The 2013 ISHLT classification resumed the concept of mixed rejection and redefined it as the coexistence of both rejections in the same endo-myocardial biopsy, underlining the possibility of cross-activation between humoral and cellular T cell immunity, precisely because the immune response to the allograft may be due to both cellular and antibody-mediated mechanisms (97). In this entity many features of ACR and severe AMR overlap, such as myocardial injury, interstitial edema, haemorrhage, polymorphic infiltrates with eosinophils and neutrophils and vasculitis with or without thrombi. The Hammond E. and Kafoury group in 2016 resumed the concept of mixed rejection with the definition written in the ISHLT classification of 2013 and demonstrated that patients with such rejection had a worse prognosis, highlighting a complex relationship between the two rejections. (97)

2 Aim of the PhD Thesis

The main aim of my thesis was to identify a miRNA signature in acute rejecting heart transplantation and determine whether assessment of miRNAs post-transplant could serve as biomarkers of heart transplant rejection. Such information should be translated in clinical practice to improve efficacy of the existing diagnostic tools.

To achieve this general aim specific goals were addressed:

a) Extraction of miRNA from formalin-fixed paraffin embedded endomyocardial biopsy (FFPE-EMBs) and recovery a good quality RNA for downstream miRNA analyses
b) Deep sequencing analysis using Ion Torrent Next Generation Sequencing platform in order to evaluate all miRNA expressed in different types of heart rejection.
c) Identification of differentially expressed miRNA (DE-miRNAs) among the sub-group and through in silico target prediction and statistical analysis and of those which could be considered as biomarkers of each rejection type
d) Validation of miRNA selected on different types of heart rejection in the FFPE-EMBs by qRT-PCR in a validation cohort
e) Analyze by in situ PCR which cell type within the FFPE-EMBs expresses the selected miRNAs.

3 Material and Methods

All solutions were prepared with ultra-pure (UP) water (Milli-Q Plus ultrapure purification, Millipore, Billerica, USA). The reagents were purchased from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland) unless stated otherwise in the text.

3.1 General equipment

Balance: Precisa 92SM-202A (Sartorius mechatronics, Göttingen, Germany)

Centrifuge: Heraeus Pico 17 (Thermoscientific, Massachusetts, USA), Heraeus Megafuge 1.0R (Thermoscientific, Massachusetts, USA), Eppendorf 5430R and Eppendorf 5415D (Eppendorf AG, Hamburg, Germany)

Microscopes: Leica-DM6000 B microscope (Leica microsystems) connected to a CCDn camera (DFC365FX, Leica microsystems)

pH-meter: WTW ph 526 (Weilheim, Germany)

Spectrophotometer: Nanodrop 1000 (PeqLab, Erlangen, Germany)

PCR thermocyclers: Thermal Cycler 7000HT fast real-time PCR system (Applied Biosystems, Germany)

Autoclave: Systec VX-95 (systec, Wettenberg, Germany)

Microtome: Leica RM2235 (Leica Biosystems, Nussloch, Germany)

Imaging software: Leica-DM6000B (Leica Biosystems, Nussloch, Germany)

Thermoblocks: Thermostat Plus (Eppendorf AG, Hamburg, Germany)

3.2 Chemical

Horse serum (Vector Laboratories, Burlingame, California, USA)

Mounting medium with DAPI (Vector Laboratories, California, USA)

Phosphate-buffered saline, PBS Dulbecco (Thermo Scientific, California, USA)

RNaseZap decontamination solution (Thermo Scientific, California, USA)

Tween® 20 (Merck, Darmstadt, Germany)

Ethanol absolute (Sigma-Aldrich, Steinheim, Germany)

BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany)

Acid-Phenol: Chloroform (Applied Biosystems, Darmstadt, Germany)

Xylol (Honeywell, Stuttgart, Germany)

3.3 Solution used in this study

20× SSC buffer: 3 M NaCl, 0.3 M Na citrate (pH 7.0).

Tris/EDTA buffer: 100 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0) (1,21 gr Tris +0,37 gr EDTA +1 L UP water + 500µl Tween 20) .

Citrate buffer: 630 ml UP water, 12.6 ml solution A (2.101 g citric acid in 100 ml UP water), 57.4ml solution B (14.70 g nitrium citrate in 500 ml of UP water), 320 µl Tween 20, (pH 6.0)

Immunofluorescence staining: Blocking solution 10% Donkey serum in PBS; Antibody Solution 0,1% Donkey serum in PBS.

3.3 Study Design and Population characteristics

I selected 33 adult patients (pts.): 20 in the study cohort and 13 in the validation cohort undergoing heart transplantation from June 2006 until July 2016 from University General Hospital of Padua (Padova, Italy). For each one I had standard graft histopathology on FFPE tissue together with DSA in the serum taken in a period near to the biopsy. Data for all patients regarding donor age, donor gender, recipient age, primary heart disease, date of transplantation, follow-up, cold ischemia time and immunosuppressive drug regimen were recorded and reported in the table 3.1. All the transplants were AB0 compatible. All participants were provided with complete information about the study and all procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human material was obtained during sampling for clinical purposes and available for research use. Surveillance EMBs included in our analyses were preferentially taken later than 2-3 weeks after transplantation to avoid the perioperative phase and were evaluated by skilled pathologists according to the last ISHLT classification for ACR (90) and to the last recommendations for the diagnosis of AMR (83) Histopathologic evaluation and C4d staining were performed on formalin-fixed, paraffin-embedded sections. For each group of rejection I selected 20 patients, who form the study cohort group, as reported in table 3.2

Table 3.1 baseline patient characteristics

	ACR >2R (n=12; 5S+7V)	pAMR >0 (n=5; 4S+1V)	MIX >2R;>0 (n=6; 5S+1V)	Control (n=10; 5S+5V)
Recipiant Age (year)	61,37	61,53	60,52	51,72
Rcipient gender (male)	9	2	3	7
Primary herat disease				
Congenital heart disease	/	/	/	2
Ischemic heart disease	7	2	2	3
arrhythmogenic cardiopathy	1	/	/	1
Valvular pathology	/	1	/	/
Dilated cardiomyopathy	4	1	1	1
Hyperthrofic cardiomyopathy	/	/	1	1
Others	1	1	1	1
Donor Age (year)	46,07±18,46	46,2±16,76	53,16±12,67	46,5±14,65
Donor Gender (male)	8	3	1	4
Cold ischemia time (min)	159±55,17	165±58,04	190±32,65	201,5±67,91
CirculatingDSA at time of biopsy	0	6	6	0
Time between TX and biopsy (day)	68	88	46,5	60

Legend: S. Study cohort; V. Validation cohort

Table 3.2 Histopathological criteria of EMBs selection

	ACR	AMR	C4d	CD68	DSA
Cellular Rejection	>= 2A-1R	-	-	-	-
Humoral Rejection	-	pAMR>0	+	+	+
Mixed Rejection	>= 2A-1R	pAMR>0	+	+	+
Control	-	-	-	-	-

Also the thirteen patients, who composed the validation group were chosen following the table 3.1

3.4 Histology and immunohistochemistry

Endomyocardial biopsies were formalin-fixed, paraffin-embedded, and routinely stained with haematoxylin-eosin. Immunohistochemistry was performed on tissue sections using affinity-purified anti-human C4d rabbit clonal antibody (Cat. No.DB 107-0.5 ;DB BIOTECH,Slovack Republic). Sections were retrieved using Citrate buffer solution on microwave (Histos 3 ; Milestone) and incubated with anti-C4d antibody at a 1/500 dilution at room temperature for 1 hour, with anti-rabbit EnVision (Dako Corporation, Hamburg, Germany) for 30 minutes and, finally, with peroxidase diaminobenzidine (DAB) for 5 minutes. Sections were counterstained with Mayer's hematoxylin for 1 minute, dehydrated in alcohol, and mounted with medium mounting (Eukitt; Bioptica). Immunohistochemical (IHC) staining of CD68, a monocyte/macrophage marker, was performed routinely and retrospectively on the paraffin sections of each biopsy, all evaluated by light microscopy. Monoclonal mouse anti-human CD68, Clone PG-M1 (dilution 1/200; Dako), was retrieved using an ethylene-diamine teraacetic aid (EDTA) solution on a microwave oven.

3.5 Assessment of donor-specific antibodies

IgG antihuman leukocyte antigens reactivity in the sera, obtained before transplantation and at the time of C4d-positive detection on EMB specimens, was analyzed using Luminex bead-based screening assays.25 and 26 Circulating anti-HLA class I/II antibodies were determined in all embs. Antibodies were considered positive if the median fluorescence intensity (MFI) was > 1000 (98)

3.6 RNA extraction: comparison between three methods

To evaluate the maximum recovery of small RNAsfrom formalin-fixed paraffin embedded endomyocardial biopsies three extraction protocols were carried out: Trizol, Phenol /chloroform plus ammonium acetate and miRNeasy FFPE kit (Qiagen) columns. RNA from formalin-fixed, paraffin-embedded tissue was shown as suitable source for miRNA expression profiling (99). From each paraffin-embedded block, 20 slides (8 µm thick-sections) were cut and collected into 2 ml microcentrifuge sterile tubes, taking care to discard the first two or three sections to avoid the possible influence of atmosphere. After dewaxing and rehydration through descending ethanol series, sections were processed for total RNA extraction using: Trizol as reported in the published protocol (100); the phenol/ chloroform according to the procedure reported by Liu et al. (101) and the miRNeasy FFPE kit (Qiagen) according to the manufacturer's instructions. Each pellet was then resuspended in a final volume of 40 µl sterile water.

3.7 Quality analysis of RNA by Agilent BioAnalyzer

All the RNA extracts were undergo to quality analysis using “Small RNA chip” by Agilet 2100 Bioanalyzer System. Small RNA chip is able to provide the percentage of enrichment in 20-40 nucleotides RNA in the samples, that are the miRNA. The Agilent 2100 Bioanalyzer System provides a better assessment of RNA intactness by showing a detailed picture of the size distribution of RNA fragments. RNA degradation is visualized with a decrease in the 18S to 28S ribosomal band ratio and an increase in the baseline signal between the two ribosomal peaks and the lower marker. Software provide a number called R.I.N., that is: RNA integrity number. This number is obtained, by software, taking the entire electrophoretic trace into account. The RIN software algorithm allows for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured.

3.8 Next Generation Sequencing (NGS) with Ion Proton

Next generation sequencing (NGS), is a sequencing technology which has revolutionised genomic research. Using NGS an entire human genome can be sequenced within a single day. In contrast, the previous Sanger sequencing technology, used to decipher the human genome, required over a decade to deliver the final draft. There are a number of different NGS platforms using different sequencing technologies, all NGS platforms perform sequencing of

millions of small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the human reference genome. Each of the three billion bases in the human genome is sequenced multiple times, providing high depth to deliver accurate data and an insight into unexpected DNA variation. NGS can be used to sequence entire genomes or constrained to specific areas of interest, including all 22 000 coding genes (a whole exome) or small numbers of individual genes.

3.8.1 Ion proton Technology

Next Generation Sequencing (NGS) technologies have dramatically increased throughput in the last five years. However, the presently available NGS technologies are too cumbersome, too slow or too expensive to sequence smaller regions of the genome. Ion Torrent sequencing technology is uniquely suited for Amplicon Sequencing because this revolutionary technology is simple, fast, scalable and cost effective.). Ion Torrent has invented the first commercially available device—a new semiconductor chip—capable of directly translating chemical signals into digital information. The sequencing chemistry itself is remarkably simple. Naturally, a proton is released when a nucleotide is incorporated by the polymerase in the DNA molecule, resulting in a detectable local change of pH. Each micro-well of the Ion Torrent semiconductor sequencing chip contains approximately one million copies of a DNA molecule. The Ion Personal Genome Machine (PGM™) sequencer sequentially floods the chip with one nucleotide after another. If a nucleotide

complements the sequence of the DNA molecule in a particular micro-well, it will be incorporated and hydrogen ions are released. The pH of the solution changes in that well and is detected by the ion sensor, essentially going directly from chemical information to digital information (Fig 2.9.1.1). If there are two identical bases on the DNA strand, the voltage is double, and the chip records two identical bases. If the next nucleotide that floods the chip is not a match, no voltage change is recorded and no base is called. Because this is direct detection—no scanning, no cameras, no light—each nucleotide incorporation is measured in seconds enabling very short run times

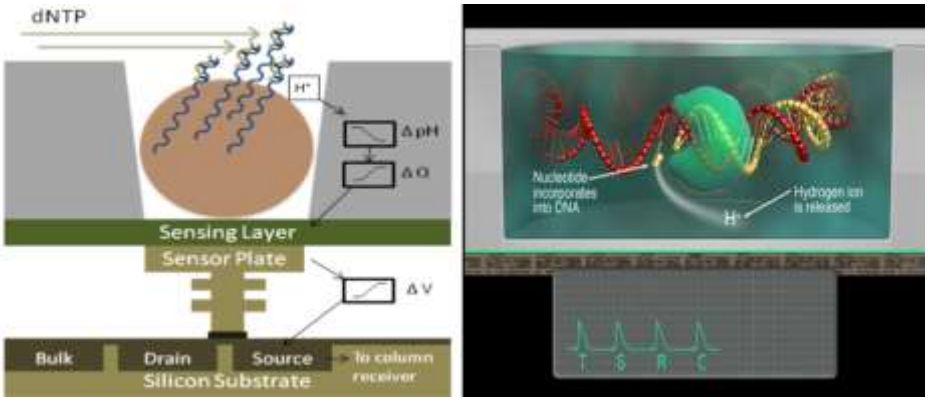


Figure 3.1 Schematic cross-section of a single well of an Ion Torrent sequencing chip*. The well houses Ion Sphere™ particles containing DNA template. When a nucleotide incorporates, a proton releases and the pH of the well changes. A sensing layer detects the chage in pH and translates the chemical signal to a digital signal.*

The first step in the workflow is to generate a library of DNA fragments flanked by the Ion Torrent adapters. This can be done by ligating the adapters to the PCR products or by adding the adapter sequences during PCR by designing PCR primers with the Ion adapter sequences at the 5' end (Figure 3.1). The library fragments are then clonally amplified onto the proprietary Ion Sphere™ particles. Clonal amplification is accomplished by emulsion PCR

(emPCR). The Ion Sphere™ particles coated with template are applied to the Ion chip. The Ion Sphere™ particles are then deposited in the chip wells by a short centrifugation step. The chip is placed on the PGM and the PGM touchscreen guides the user to set up the sequencing run. Once data is generated on the Ion PGM™ sequencer, it is automatically transferred to the required Torrent Server. Here data are run through signal processing and base calling algorithms that produce the DNA sequences associated with individual reads.

Figure 3.2. Schematic representation of the Ion Torrent sequencing workflow. *A sequencing library is produced by generating DNA fragments flanked by the Ion Torrent sequencing adapters. These fragments are clonally amplified on the Ion Sphere™ particles by emulsion PCR. The Ion Sphere™ particles with the amplified template are then applied to the Ion Torrent chip and the chip is placed on the Ion PGM™. The sequencing run is set up on the Ion PGM™. Sequencing results are provided in standard file formats. Downstream data analysis can be performed using the DNA-Seq workflow of the Partek® Genomics Suite™*

3.8.2 Library production and Sequencing

Total RNA was enriched in small RNA with Agencourt AMPure XP beads (Beckman Coulter) according to the Ion Total RNA-Seq v.2 kit (IonTorrent, Thermo-Fishcher Scientific); the percentage of miRNA among all other kinds of small RNA was assessed with Agilent Small RNA Analysis kit on the Agilent Bioanalyzer (Agilent Technologies). Then, for each sample, we

processed a quantity of small RNA enriched containing 25 ng of miRNA and proceeded with library construction according to Ion Total RNA-Seq v.2 kit (IonTorrent, Thermo-Fishcher Scientific) protocol. Briefly, 3' and 5' directional adapters were ligated to RNA molecules prior to reverse transcription, cDNA was then amplified with barcoded primers. Samples were loaded on Ion PI chip v.3 of the Ion Proton Sequencer (IonTorrent, Thermo-Fishcher Scientific)

3.8.3 Sequencing and statistical analysis

The produced reads were analyzed with catadapt (102) in order to remove adapter sequences that could be still present after sequencing. Reads shorter than 15 bp after adapter removal where discarded. Passing filters reads were aligned using PASS aligner allowing identity of at least 94% (103) gainst miRBase database(104) . Counts were produced counting reads aligning on each single miRNA with a strategy developed to avoid the over-estimation: multi-mapping reads, for example read aligning to a miRNA coming from different loci, were counted as single read and not as multiple alignment.

After removal of miRNA having average read count, among all samples, lower than 10, read counts were normalized with the Trimmed Mean of M-values method [Robinson MD, Oshlack A, 2010]. Differentially expressed miRNA were identified using the common dispersion model within the edgeR package of the Bioconductor R framework (105) with a False Discovery Rate lower than 0.05.

3.9 cDNA synthesis and quantitative real-time RT-PCR analysis

The differential regulation of the miRNAs was studied by quantitative PCR (qRT-PCR) which runs on a 7000HT thermocycler. SYBR green expression assays were used in all the experiments. SYBR green is a DNA binding dye that binds to all double stranded DNA during PCR and results in fluorescence which can be measured. It is important to have well-designed SYBR green primers that do not amplify non-target sequences. Primers for qRT-PCR used were list in table 3.3. Primers were purchased by Qiagen.

Table 3.3: Primers for qRT-PCR.

Name of miRNA	miRNA sequence 5'-3'
hsa-miR-190a-5p	5'UGAUAUGUUUGAUAUAUUAGGU
hsa-miR-218-5p	5'UUGUGCUUGAUCUAACCAUGU
hsa-miR-31-5p	5'AGGCAAGAUGCUGGCAUAGCU
hsa-miR-451a	5'AAACCGUUACCAUUACUGAGUU
hsa-miR-135a-5p	5'UAUGGCUUUUUAUUCCUAUGUGA
hsa-miR-144-3p	5'UACAGUAUAGAUGAUGUACU
hsa-miR-126-5p	5'CAUUAUUACUUUUGGUACGCG
hsa-miR-29b-3p	5'UAGCACCAUUUGAAAUCAGUGUU
hsa-miR-29c-3p	5'UAGCACCAUUUGAAAUCGGUUA
hsa-miR-199a-3p	5'ACAGUAGUCUGCACAUUGGUUA
hsa-miR-27b-3p	5'UUCACAGUGGCUAAGUUCUGC
hsa-miR-208 a-5p	5'GAGCUUUUGGCCCGGGUUAUAC

In this technique, the amplified cDNA is measured as the reaction progresses. During PCR, an increase in the DNA product leads to an increase in fluorescence intensity which can be measured at each cycle and hence the DNA concentration is quantified. This in turn can be used to calculate the expression of a target gene. Total RNA was isolated from paraffin embedded sections using miRNeasy Mini Kit (Qiagen), as reported in the 3.7 section. For qRT-PCR, RNA concentration was determined by measuring the absorbance at 260 nm (A260) using spectrophotometer. The absorbance at 280 nm was also measured to determine the RNA purity. RNA with an A260/A280 ratio of 1.8-2.0 was used. RNA was reverse transcribed with the reverse transcription master mix (miScript II RT Kit, Qiagen, Hilden, Germania) according to the manufacturer's protocol (Table 3.4). The RT reaction consists of the following steps: 37°C for 60 min, 95°C for 5 min, and 4°C.

Table 3.4. Reverse transcription

Solution	Volume
5x miScript HiSpec Buffer	2 µl
10x miScript Nucleics Mix	1 µl
miScript Reverse Transcriptase Mix	1 µl
Nuclease-free water	Variable
RNA (200 ng)	3--5 µl
Total volume	12,5 µl

The cDNA was subjected to PCR amplification with specific set of SYBR green primers (Qiagen, Table 3.3). PCR was performed in a 7000HT fast real-time PCR system (Applied Biosystems, Darmstadt, Germany) (Table 3.5).

Table 3.5 Real-time PCR program

Steps	Temperature	Time	
Initial denaturation	95° C	15 min	
Denaturation	94° C	15 sec	←
Annealing	55° C	30 sec	40
Extension	70° C	30 sec	

The relative expression levels were calculated using multiple internal control genes, adjusted for differences in PCR efficiency (Qbase, Biogazelle, Zwijnaarde, Belgium) and logarithmically transformed. In order to obtain accurate measurement of gene expression, multiple internal control genes were used and the analysis was performed using the Qbase software. This software calculates the expression stability of the housekeeping genes in different samples. This is based on the principle that the ratio of two housekeeping gene expression is identical among all the samples (106). An increasing variation in the ratio corresponds to decreasing expression stability of the housekeeping genes. The relative expression levels were normalized to the reference genes and logarithmically transformed (log10) (Qbase, Biogazelle).

3.10 Combined In situ PCR and Immunostainings

In situ PCR is a method for the miRNAs detection in formalin fixed, or paraffin-embedded tissues. This method involves the extension of the labeled miRNA hybridized to a template with 100-nucleotide–long ultramer that contains the complementary sequence of the miRNA 3′-UTR. The extension method results in visualizing the miRNA signal in specific cells and tissues with using RT-PCR (107).

Formalin fixed paraffin embedded endo-myocardial biopsy sections (4 µm thick) were deparaffinised following the protocols describe in table 3.6

Table 3.6. Deparaffinization protocol for *in situ* PCR.

Step	Reagent	Time
1	Xylene	5 min
2	Xylene	5 min
3	100% Ethanol	3 min
4	100% Ethanol	3 min
5	96% Ethanol	3 min
6	70% Ethanol	3 min
7	100% Ethanol	5 s
8	air-dry	2 min

After deparaffinizzation the section were treated with Tris ethylene-diamine tetra-acetic acid (EDTA) added with Tween 20 solution for the antigen

retrieval and cooked 3 times for 10 min each on a microwave oven regulated at 600 W .

Sections were incubated with DNase (Roche, Basel, Switzerland) ON at 37°C. For each sample were added 1µl DNase, 5µl 10x incubation buffer, 44µl RNase-free water (Figure 3.3).

Figure 3.3*. Approximately 50 µL of the DNase was added to each section through the hole of the hybridization chamber and covered RNase-free adhesive film. The slides were then incubated at 37 °C in the Thermoblocks or Thermal cyclers.*

One-step reverse transcriptase in situ PCR was performed in MasterCycler equipped with an adjustable slide container using gene-specific PCR in situ primers (Sigma-Aldrich, Table 3.7), SuperScript One-Step RT-PCR with PlatinumTaq (Thermo Scientific) and digoxigenin-11-dUTPs (Roche) (107) (108). The worm-specific microRNA cel-miR-39 was used as negative control.

Table 3.7 *In situ* PCR Primers sequences.

Gene	Primer sequences 5'-3'
Ultramer hsa miR-144-3p:	GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTC TAAAGACCCCTTAATGCGTCTAAAAGTACATCATCT ATACTGTA
Ultramer hsa-miR-29b-3p :	GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTC TAAAGACCCCTTAATGCGTCTAAAAACACTGATTTC AAATGGTGCTA
Ultramer hsa-miR-126-5p	GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTC TAAAGACCCCTTAATGCGTCTAAA CGCGTACCAAAAGTAATAATG
Ultramer hsa-miR-451a:	GACCCCTTAATGCGTCTAAAGACCCCTTAATGCGTC TAAAGACCCCTTAATGCGTCTAAA AACTCAGTAATGGTAACGGTTT
Taq-in situ-cel-miR-39–Reverse-Transcription	GTCGTATCCAGTGCAGGGTCCGAGG TATTCGCACTGGATACGACCAAGC

Two serial sections were placed on the same slide, one of which was used for negative control staining. SecureSeal™ hybridization chambers were attached to the slides and 50 µL reaction mix (Table 3.8) was added through access ports to the microwells formed by the hybridization chambers. The slides were then placed in a thermal cycler (Eppendorf Master Cycler Nexus) and reverse transcription and amplification was performed (Table 3.9). After completing the cycles, slides were rinsed first with xylene and then with ethanol (100%), before air-drying.

Table 3.8 *In situ* reverse transcriptase PCR reaction mix

Reagents	Volume (μL)
Platinum Taq DNA Polymerase (SuperScript One-Step RT-PCR System)	1
2xReaction mix buffer (SuperScript One-Step RT-PCR System)	25
2% BSA	1.6
Digoxigenin-11-dUTPs (1 mM)	0.6
RNase inhibitor	1.4
Um-miR-Primer (250 μM)	2
RNase-free water	18.4

Table 3.9. Thermal cycling conditions.

Steps	Cycles	Temperature	Time
1	1	55° C	35 min

After washing with SSC buffer and blocking the nonspecific binding sites using nitroblue tetrazolium chloride (PerkinElmer, MA, USA) and biotin/avidin binding sites by using a blocking kit (Vector Laboratories), sections were incubated for 1 h at 37°C with a peroxidase-conjugated anti-digoxigenin sheep F'ab fragments (Fab fragments from sheep, 1:100 dilution; Roche). To visualize the probe a tyramide-based amplification system (TSA Plus Biotin, PerkinElmer) and Dylight 549–conjugated streptavidin (1:200)

were used. Sections were subsequently stained with different types of antibodies. Antibodies used were listed in Table 3.10.

Table 3.10 Primary Antibodies in use in this study

Antigen	Clone	Host	Diluition	Antigen retrieval	Catalogue #	Company
α-SMA	1A4	mouse	1:200	Tris-EDTA 30 min (100)°C	M0851	Dako, Hamburg, Germany
Cardiac Troponin T	polyclonal	goat	1:300	Tris-EDTA 30 min (100)°C	ab64623; SAP 566773	Abcam, Cambridge, UK
human von Willebrand Factor	polyclonal	rabbit	1:1000	Tris-EDTA 30 min (100)°C	ab6994	Abcam, Cambridge, UK
CD3	Monoclonal Clone F7.2.38	mouse	1:200	Tris-EDTA 30 min (100)°C	M7254	Dako, Hamburg, Germany

CD68	monoclonal	mouse	1:200	Tris-EDTA 30 min (100)°C	ab955; SAP 603504	Abcam, Cambridge, UK
Antigen	**Clone**	**Host**	**Dilution**	**Antigen retrieval**	**Catalogue #**	**Company**
Nonspecific primary Ab	monoclonal	mouse	1:100	Tris-EDTA 30 min (100)°C	sc-2025	Santa Cruz Biotechnology
Nonspecific primary Ab	monoclonal	rat	1:100	Tris-EDTA 30 min (100)°C	sc-2026	Santa Cruz Biotechnology
Nonspecific primary Ab	polyclonal	rabbit	1:100	Tris-EDTA 30 min (100)°C	ab27472	Abcam
Nonspecific primary Ab	polyclonal	goat	1:100	Tris-EDTA 30 min (100)°C	sc-2028	Santa Cruz Biotechnology

For in situ PCR immunohistochemistry the antibodies combinations reported in table 3.11

Table 3.11. Antibodies combination for PCR in situ immunohistochemistry

Priamary Antibody	Diluition Factor	Secondary Antibody	Diluition Factor
SMA	1:100 in Ab solution	α-Mouse-FITC coniugated	1:100 in PBS
Troponin	1:200 in Ab solution	α Goat Cy5 Coniugated	1:100 in PBS
CD 3	1:100 in Ab solution	α Rat Cy5 coniugated	1:100 in PBS
CD 68	1:200 in Ab solution	α Mouse FITC Coniugated	1:100 in PBS
vWF	1:1000 in Ab Solution	α Rabbit FITC Coniugated	1:100 in PBS
Troponin	1:200 in Ab solution	α Goat Cy5 Coniugated	1:100 in PBS

The antibodies combination for Isotype negative control is reported in table 3.12

Table 3.12 Isotype negative control Combination

Priamary Antibody	Diluition Factor	Secondary Antibody	Diluition Factor
Normal Mouse IgG	1:100 in Ab solution	α-Mouse-FITC coniugated	1:100 in PBS
Normal Goat IgG	1:100 in Ab solution	α Goat Cy5 Coniugated	1:100 in PBS
Normal Rat IgG	1:50 in Ab solution	α-Rat-FITC coniugated	1:100 in PBS
Normal Goat IgG	1:100 in Ab solution	α Goat Cy5 Coniugated	1:100 in PBS

The fluorescent signals were acquired by Leica-DM6000 B microscope (Leica microsystems) connected to a CCD camera (DFC365FX, Leica microsystems) using the following filters:

Dapi: dc400 excitation: 340-380 emission:450-490;

Green filter (FITC) excitation:460-500 emission: 512-542;

Yellow filter (Cy3): excitation: 540-552 emission: 580-620;

far Red (Cy5): excitation : 590-650 emission: 662-738

4 Results

4.1 RNA extraction: Comparison of the three methods based on quantity and quality results.

Archived formalin-fixed paraffin-embedded (FFPE) tissue samples represent excellent resources for biomarker discovery (99). In general, the different methods are based on the use of: "classic" reagents for nucleic acid extraction, such as guanidine isothiocyanate and phenol (TRIzol or TRI Reagent) solutions or silica-based columns. In order to evaluate the best extractive technique to be applied to endomyocardial biopsies, 3 types of extraction were carried out: Trizol, Phenol /chloroform plus ammonium acetate and miRNeasy FFPE kit (Qiagen) columns. The data relative of the three types of extraction are reported in the table 4.1

	260/230	260/280	Conc. ng/µl	RIN
Phenol/ Chloroform	0.845	1.727	40,8	1.9
	0.873	1.524	37,8	2.2
	0.975	1.706	52	2.8
Trizol	1.789	1.373	34	1.9
	1.468	1.658	37	2.2
	1.649	1.725	14	2.5
miRNeasy FFPE kit (Qiagen)	2.000	1.818	88,4	3.7
	1.92	1.727	66,4	3.5
	1.71	2.266	93.2	3.6

Table 4.1:Quality parameters, concentrations (ng / µl)and RIN of the samples according to the type of extraction examined according to the type of extraction.

The extracts have concentrations between 14 and 88,4 ng / µl; as can be seen from the data in Table 4.1 the best yields are obtained with the extraction by miRNeasy Kit (Qiagen), although also the extraction with the phenol / chloroform shows good yields. Furthermore, all samples extracted with Qiagen Kit show A260 / A280 ratios between 1.8-2 and A260 / A230 ranging from 1.8-2.2 to demonstrate that the RNA is of good quality; while in the samples extracted with the phenol and Trizol they have ratios A260 / A280 and A260 / A230 lower than 1.8 because of a probable contamination from proteins and from phenol.

These analyses led us to choose the use of columns kit as extraction method.

To test the success of the extraction, I first performed a NanoDrop ND1000 reading. This instrument is a very compact spectrophotometer which, in addition to providing total RNA concentrations, gives a rough indication of the purity of the sample. In order to discriminate between the various RNA fractions present in our extractions, a run was performed on the Agilent 2100 bioanalyzer, an analyzer using microfluidic technology for biological sample analysis by the use of specific chips and reagents for analysis of fragments of small RNA. Each chip contains a set of interconnected micro channels that is used to split the fragments by size.

This device check RNA quality with RIN (RNA Integrity Number), the standard for RNA analysis that provides total RNA, mRNA and Small RNA data. The recommended concentration for purified samples with RNA ≤ 150 nt is 1-20 ng / µl. The bioanalyzer creates an electropherogram for each sample in which it is possible to distinguish the peaks where miRNAs are present (between 10 and 40nt) and have the percentage of miRNA present. In the figure 4.1 I show a representative electropherogram of one sample.

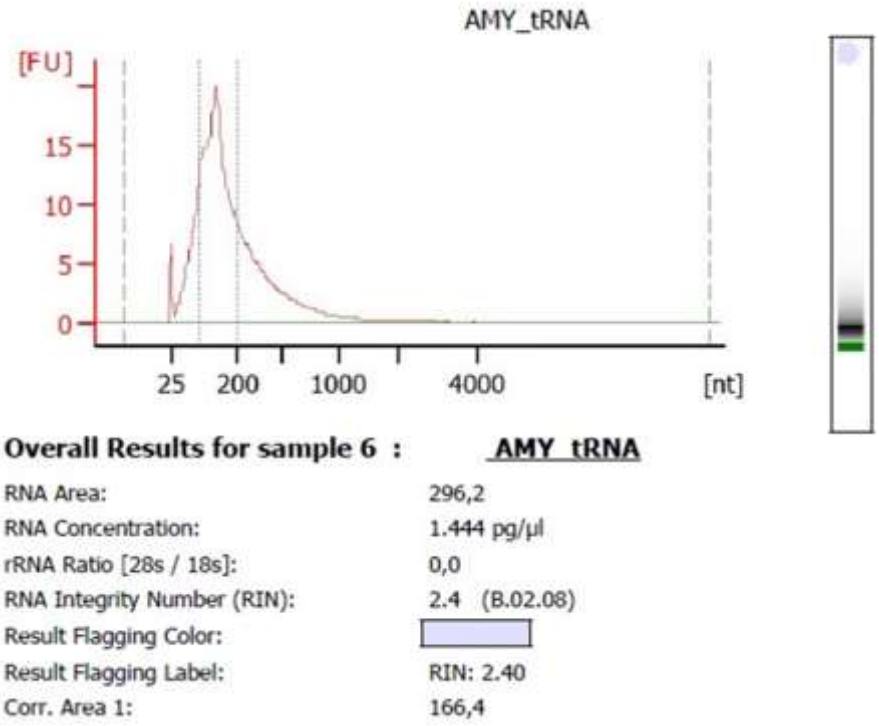


Figure 4.1. Representative total RNA integrity analysis using Agilent 2100 bioanalyzer.

The RIN value for all my specimens is included between 2-3 as reported in literature for this sample (109) therefore the RNA can be used for the downstream analysis without any negative impact.

4.2 Whole miRNAs expression in study cohort.

In order to identify clusters of miRNAs which may be involved in the rejection after heart transplantation, a NGS experiment with Ion Proton technology approach was used to investigate global miRNAs expression patterns. In this regard, I analysed the expression of all mature miRNAs.

19 patients were divided into 4 groups on the base of rejection diagnosis:

- ACR 5 pts.
- AMR 4 pts.
- MIX 5 pts.
- Control 5pts.

This analysis had the potential to identify differentially expressed miRNAs (DE-miRNAs) that could be used as diagnostic biomarkers for rejection in the tissue and could be targeted as new therapeutic targets. To have a general view of the overall detected and DE-miRNAs in the examined tissues, I performed the analysis by a NGS with semiconductor approach, that allows the detection of even small differences among samples, and enables the identification of annotated miRNAs. After small-RNA seq experiment, a multiple repetition of cutadapt was used to remove all adapters and technical sequence artefacts. The cleaned sequences were mapped to mature miRNA and snoRNA with bowtie2 in local mode (allowing for soft clipping of reads at the ends) and uniquely matching reads with no mismatches were selected. The NGS analysis generated a list of differentially expressed miRNAs in tissues representing a valuable framework to better understand the abnormalities of cellular pathways associated with the pathogenesis of rejections, and to identify candidates for therapeutic intervention. In the heat-maps are shown all DE-miRNAs that I found by NGS. The colours show the levels of alteration of each DE-miRNA (in blue downregulated miRNAs, in red upregulated miRNAs); the brilliance of colours is proportionally to the grade of alteration. Differentially expressed miRNA were identified using the common dispersion model within the edgeR package of the Bioconductor R framework with a False Discovery Rate lower

than 0.05. During the sequencing one sample of the pAMR group was not adeguate and was excluded it from the subsequent analyses .

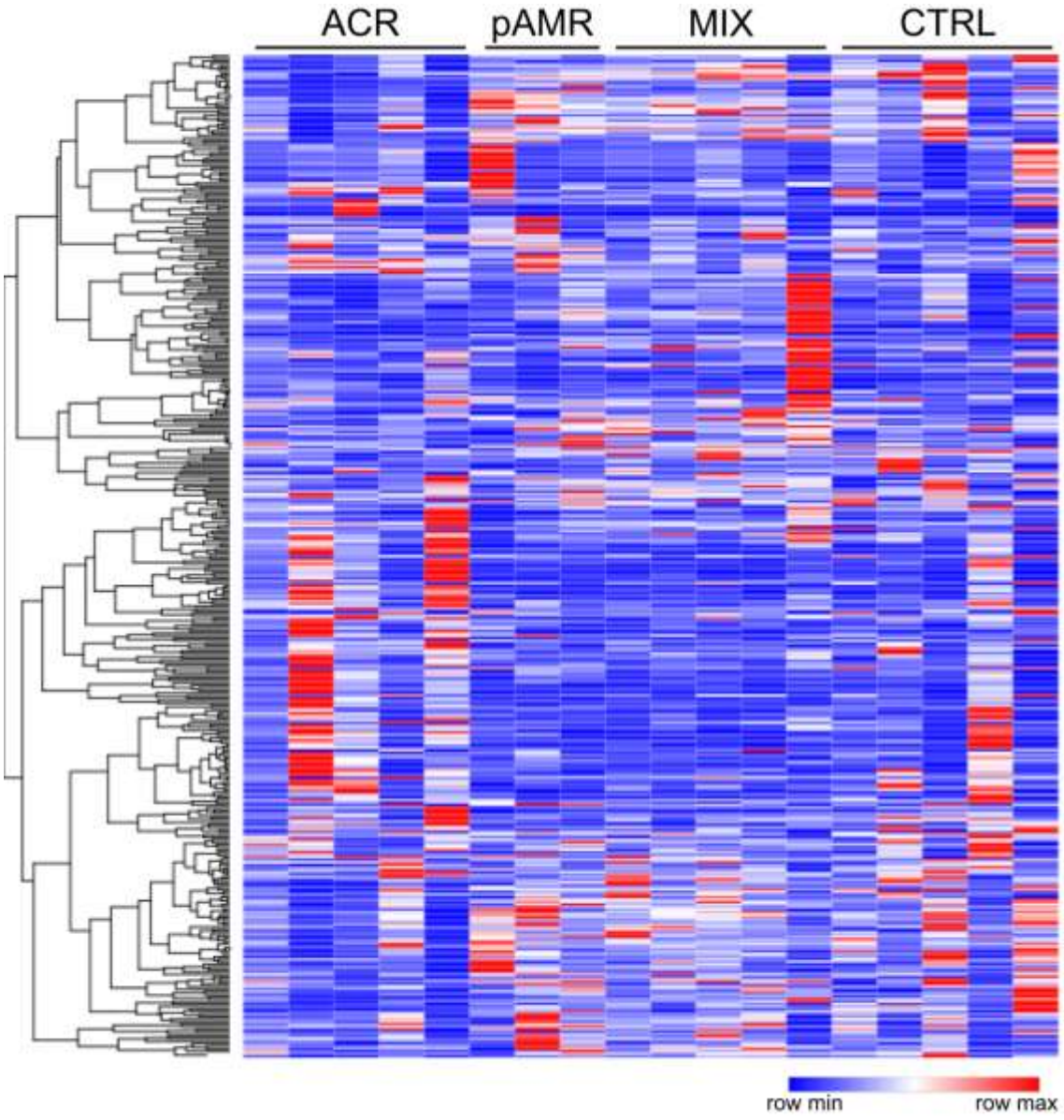
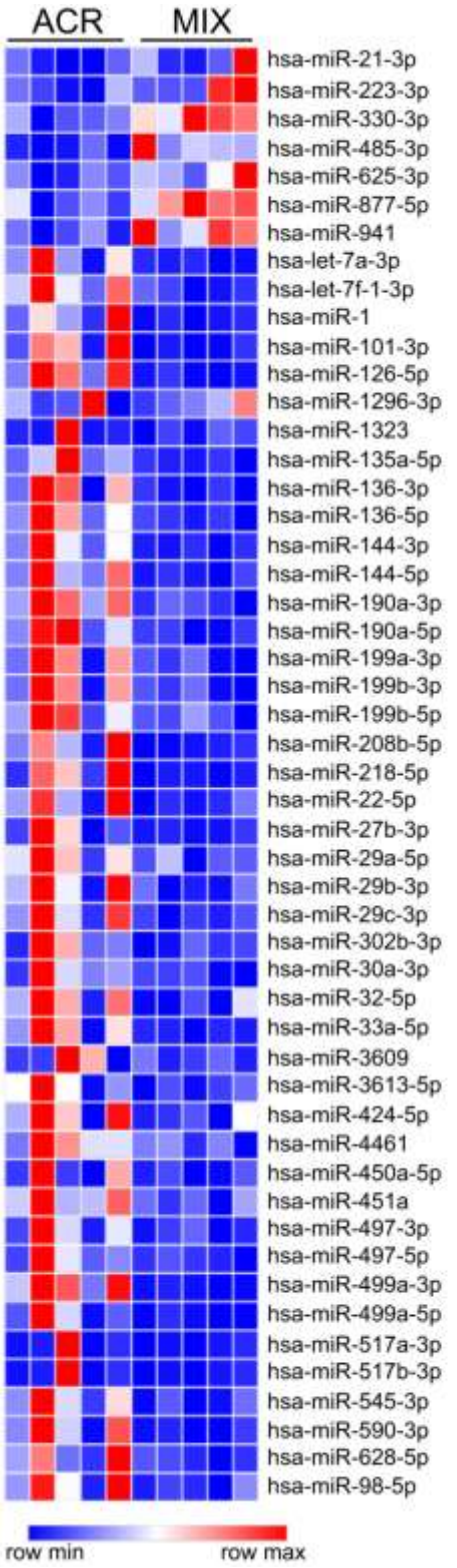


Figure 4.2. **Global miRNAs expression and unsupervised hierarchical clustering of the 3 rejection types and Control group**. The miRNAs expression value showed in the map is Log of the original value. Each row represents the expression level of miRNAs, and each column is tissue sample. The color scale setting is according to the MultiExperiment Viewer v4.2 software's indication from -0.5 to 4.8.

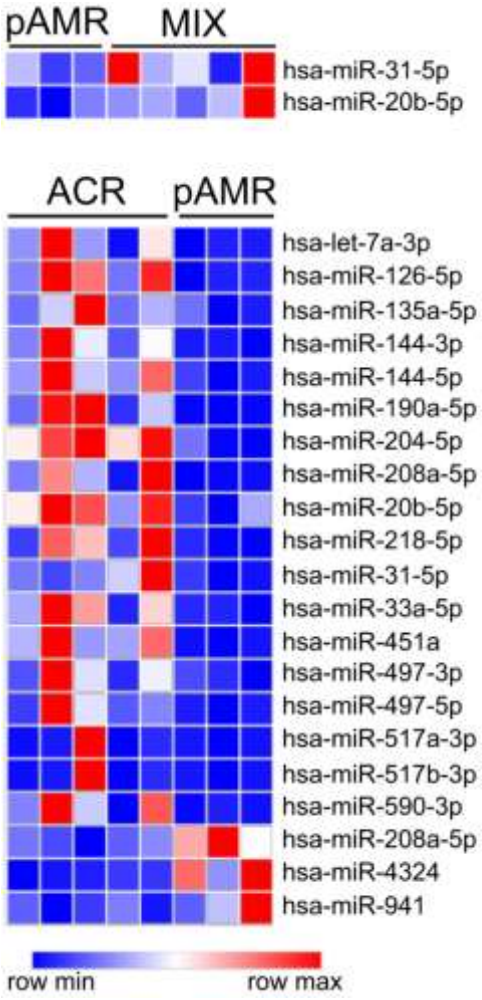
4.2.1 Comparison of miRNAs in rejection types.

The three types of rejection and control groups were compared in pair with the un-supervised analysis showing a typical profile of differentially expressed miRNAs for each group; in particular: Mixed vs AMR: only 2 miRNAs overexpressed in the Mixed group suggesting a similarity between the two types. ACR vs AMR: 18 miRNAs overexpressed and 2 miRNAs under-expressed in the ACR. Mixed vs ACR : 7 miRNAs under expressed and 39 miRNAs over-expressed in the ACR group.



miRNA	ACR vs. MIX (FC)	p value
hsa-miR-21-3p	-2,9	0,020
hsa-miR-223-3p	-3,11	0,010
hsa-miR-330-3p	-3,32	0,010
hsa-miR-485-3p	-3,1	0,020
hsa-miR-625-3p	-2,55	0,050
hsa-miR-877-5p	-2,65	0,040
hsa-miR-941	-3,54	0,010
hsa-let-7a-3p	7,06	3,759E-08
hsa-let-7f-1-3p	3,35	0,012
hsa-miR-1	2,53	0,031
hsa-miR-101-3p	2,75	0,016
hsa-miR-126-5p	4,42	0,0002
hsa-miR-1296-3p	3,55	0,008
hsa-miR-1323	3,53	0,005
hsa-miR-135a-5p	4,87	7,50E-05
hsa-miR-136-3p	5,06	0,0009
hsa-miR-136-5p	4,24	0,001
hsa-miR-144-3p	6,07	3,759E-06
hsa-miR-144-5p	3,88	0,001
hsa-miR-190a-3p	3,39	0,008
hsa-miR-190a-5p	5,08	4,153E-05
hsa-miR-199a-3p	2,43	0,042
hsa-miR-199b-3p	2,45	0,040
hsa-miR-199b-5p	2,86	0,012
hsa-miR-208b-5p	3,59	0,002
hsa-miR-218-5p	3,95	0,001
hsa-miR-22-5p	2,61	0,026
hsa-miR-27b-3p	2,38	0,047
hsa-miR-29a-5p	2,81	0,028
hsa-miR-29b-3p	2,67	0,021
hsa-miR-29c-3p	2,50	0,033
hsa-miR-302b-3p	3,55	0,004
hsa-miR-30a-3p	2,57	0,033
hsa-miR-32-5p	2,68	0,026
hsa-miR-33a-5p	4,40	0,001
hsa-miR-3609	3,00	0,028
hsa-miR-3613-5p	3,58	0,004
hsa-miR-424-5p	2,41	0,045
hsa-miR-4461	2,93	0,016
hsa-miR-450a-5p	3,23	0,012
hsa-miR-451a	2,73	0,017
hsa-miR-497-3p	3,97	0,001
hsa-miR-497-5p	3,62	0,001
hsa-miR-499a-3p	5,56	1,119E-05
hsa-miR-499a-5p	5,95	3,759E-05
hsa-miR-517a-3p	8,88	3,148E-07

Figure 4.3. **Differential expressed miRNAs in the comparison ACR vs MIX**. The miRNA expression value showed in the map is Log of the original value. ACR vs MIX: 7 miRNAs under expressed and 39 miRNAs over-expressed in the ACR group; The table show the corresponding fold change and P-value of each microRNAs de-regulated in the comparison.



miRNA	pAMR vs MIX (FC)	p value
hsa-miR-31-5p	-7,23	0,006
hsa-miR-20b-5p	-4,83	0,016

miRNA	pAMR vs MIX (FC)	p value
hsa-let-7a-3p	7,46	0,004
hsa-miR-126-5p	4,45	0,019
hsa-miR-135a-5p	4,18	0,034
hsa-miR-144-3p	6,85	0,004
hsa-miR-144-5p	8,5	0,004
hsa-miR-190a-5p	4,43	0,022
hsa-miR-204-5p	5,03	0,019
hsa-miR-208b-5p	4,72	0,019
hsa-miR-20b-5p	4,5	0,022
hsa-miR-218-5p	5,92	0,006
hsa-miR-31-5p	5,15	0,022
hsa-miR-33a-5p	8,14	0,006
hsa-miR-451a	3,88	0,029
hsa-miR-497-3p	6,46	0,019
hsa-miR-497-5p	4,17	0,022
hsa-miR-517a-3p	7,83	0,006
hsa-miR-517b-3p	7,83	0,006
hsa-miR-590-3p	4,65	0,022
hsa-miR-208a-5p	-3,51	0,044
hsa-miR-4324	-3,57	0,022
hsa-miR-941	-4,26	0,019

Figure 4.4. **Differential expressed miRNAs in the comparison pAMR vs MIX and ACR vs pAMR.** The miRNA expression value showed in the map is Log of the original value.
AMR vs MIX: only 2 miRNAs overexpressed in the MIX. ACR vs AMR: 18 miRNAs overexpressed and 3 miRNAs under-expressed in the ACR. The tables show the corresponding fold change and P-value of each microRNAs de-regulated in the comparison.

The analysis revealed that there are microRNAs de-regulated between the three rejections confirming our hypothesis that microRNAs can characterize the three pathological conditions.

4.3 Selection of microRNAs as possible biomarkers.

MiRNAs have been selected for further evaluation and validation, based on the number of reads resulting by NGS, on their highly significant FDR (< 0.05)

and most importantly on their fold change and p-value. I selected those microRNAs that showed a fold change > ± 2.5 in each comparison. I did not consider , in this first step, those microRNAs that affect genes involved in cancers or others pathologies, other ones have no validated targets and could be misleading to achieve the goal of my thesis . Therefore I focused on those microRNAs known to be involved and validated to target genes implicated in typical pathways of rejection as shown by a bioinformatic analysis and reported in public databases such as TarBase (version 6.0) (110) , miRTarBase (111) , miRWalk (112), miRecords (113), DIANA-microT-CDS (114) , miRmap (115), miRDB (116) , TargetScan (117), and miRanda (118). The comparative analysis of miRNA levels across rejection types was performed with edgeR, a statistical package based on generalized linear models, suitable for multifactorial experiments. The potential role, the target genes, and the pathways in which DE-miRNA identified by NGS were involved, were analysed by bioinformatics tools (Gene Ontology, KEGG frequencies) and literature-based analysis (PubMed). As shown in the figures below some of these DE-miRNAs are already known to modulate the expression of genes involved in pathways such as immune system cells cycle regulation and proliferation, inflammatory pathways NFkB mediated and endothelial remodelling that may be relevant for rejection pathophysiology. Some of them are known to modulate the expression of genes involved in more than one of these pathways because they are predicted to target relevant genes involved in important processes.

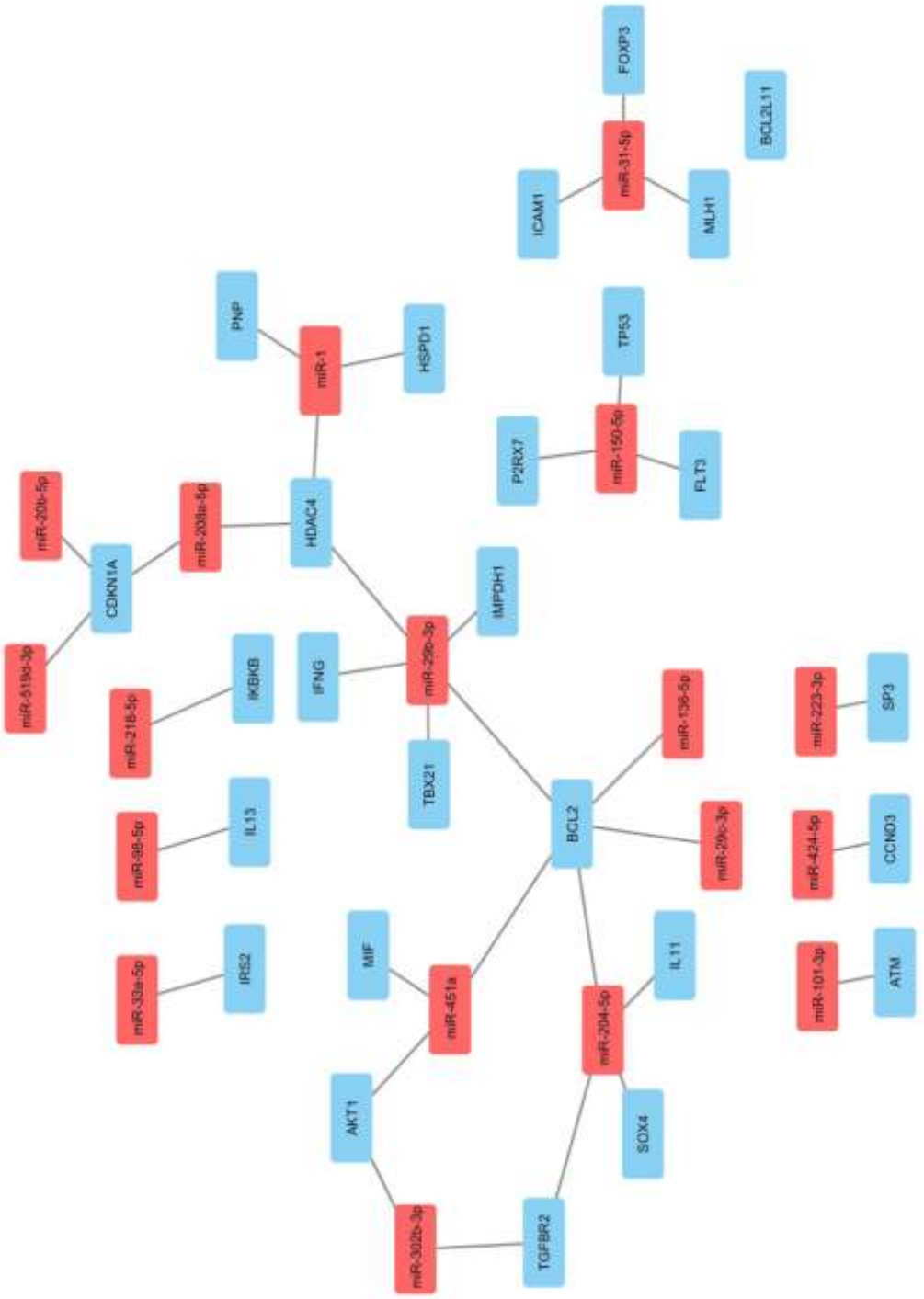


Figure 4.5 microRNAs experimentally validated to target genes involved in the regulation of Lymphocytes activation and proliferation. Integrative target prediction analysis by Cytoscape using DE- miRNAs among comparison. In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

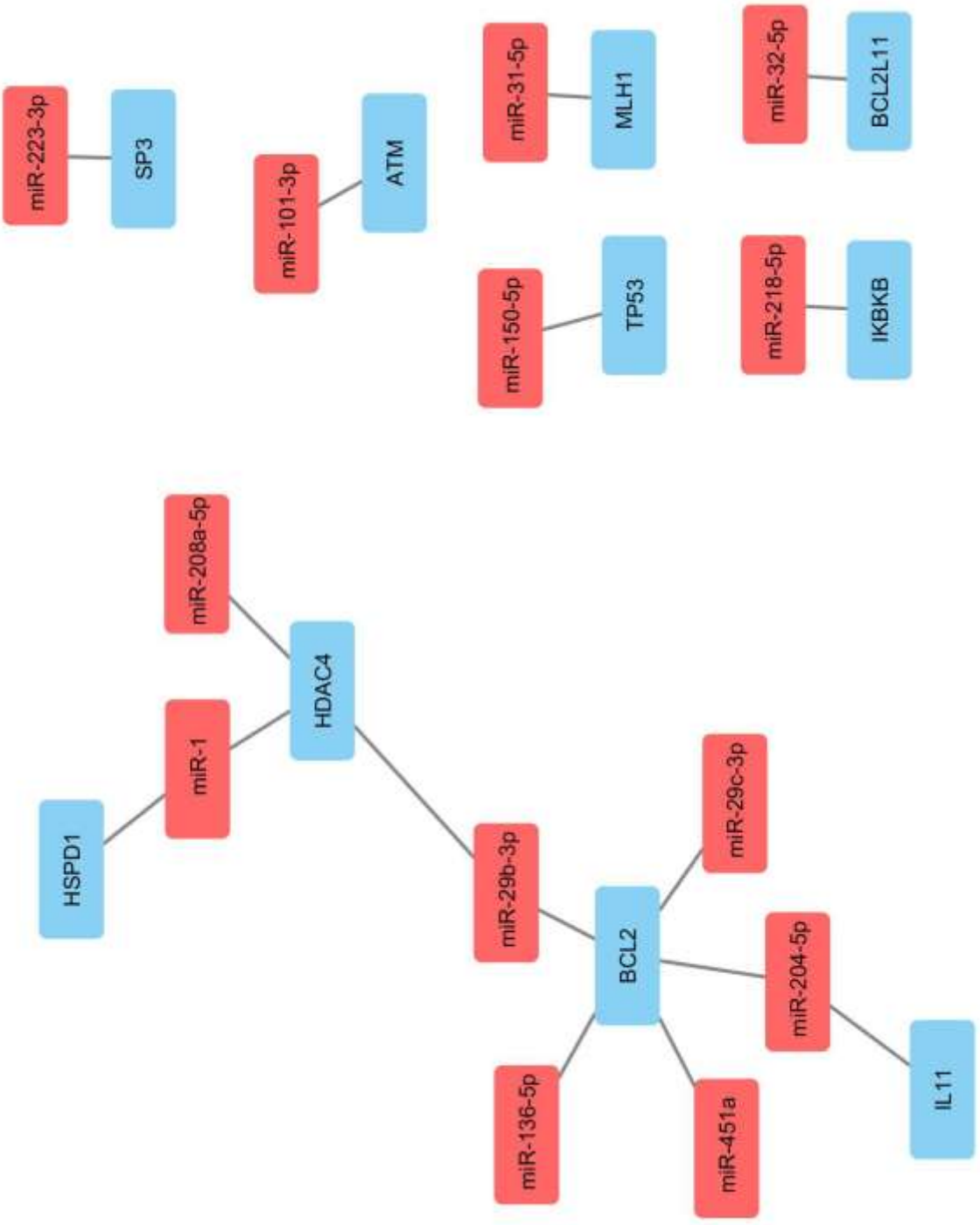


Figure 4.6 microRNAs experimentally validated to target genes involved in the regulation of B-cell Activation. Integrative target prediction analysis by Cytoscape using DE- miRNAs among comparison.In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

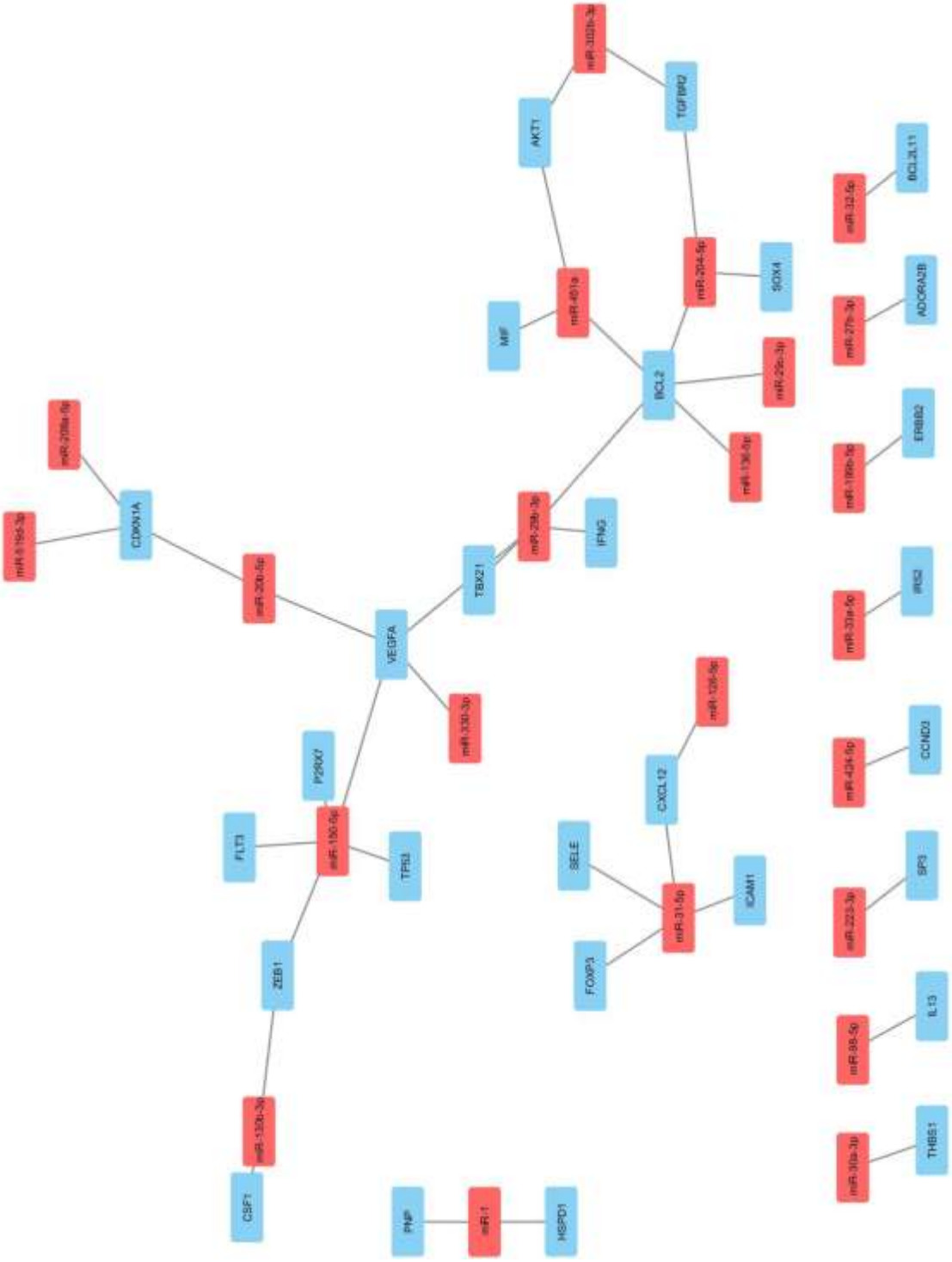


Figure 4.7 microRNAs experimentally validated to target genes involved in the regulation of T-cell Activation. Integrative target prediction analysis by Cytoscape using DE- miRNAs among comparison. In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

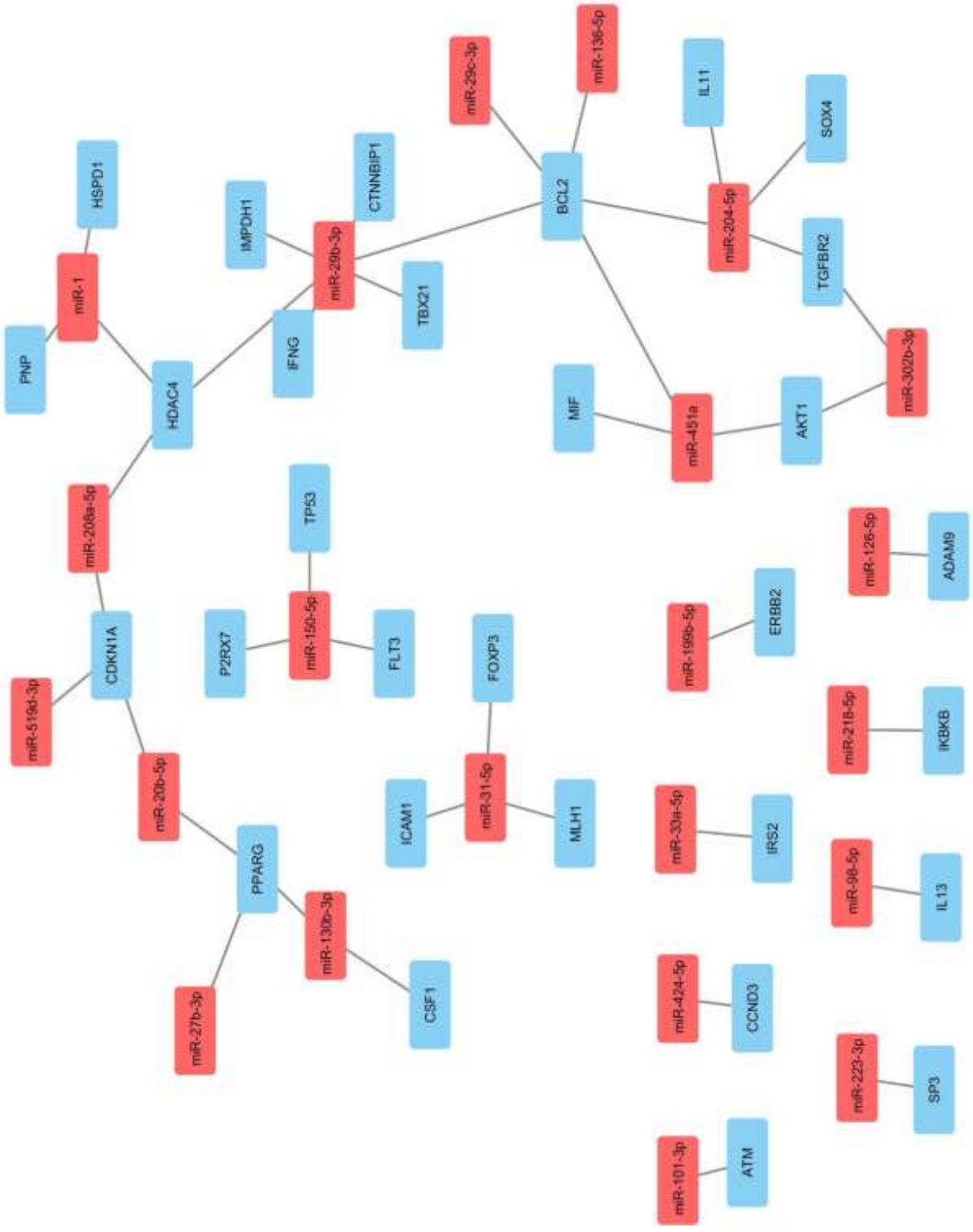


Figure 4.8 microRNAs experimentally validated to target genes involved in the regulation of Monocytes Activation. Integrative target prediction analysis by Cytoscape using DE-miRNAs among comparison. In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

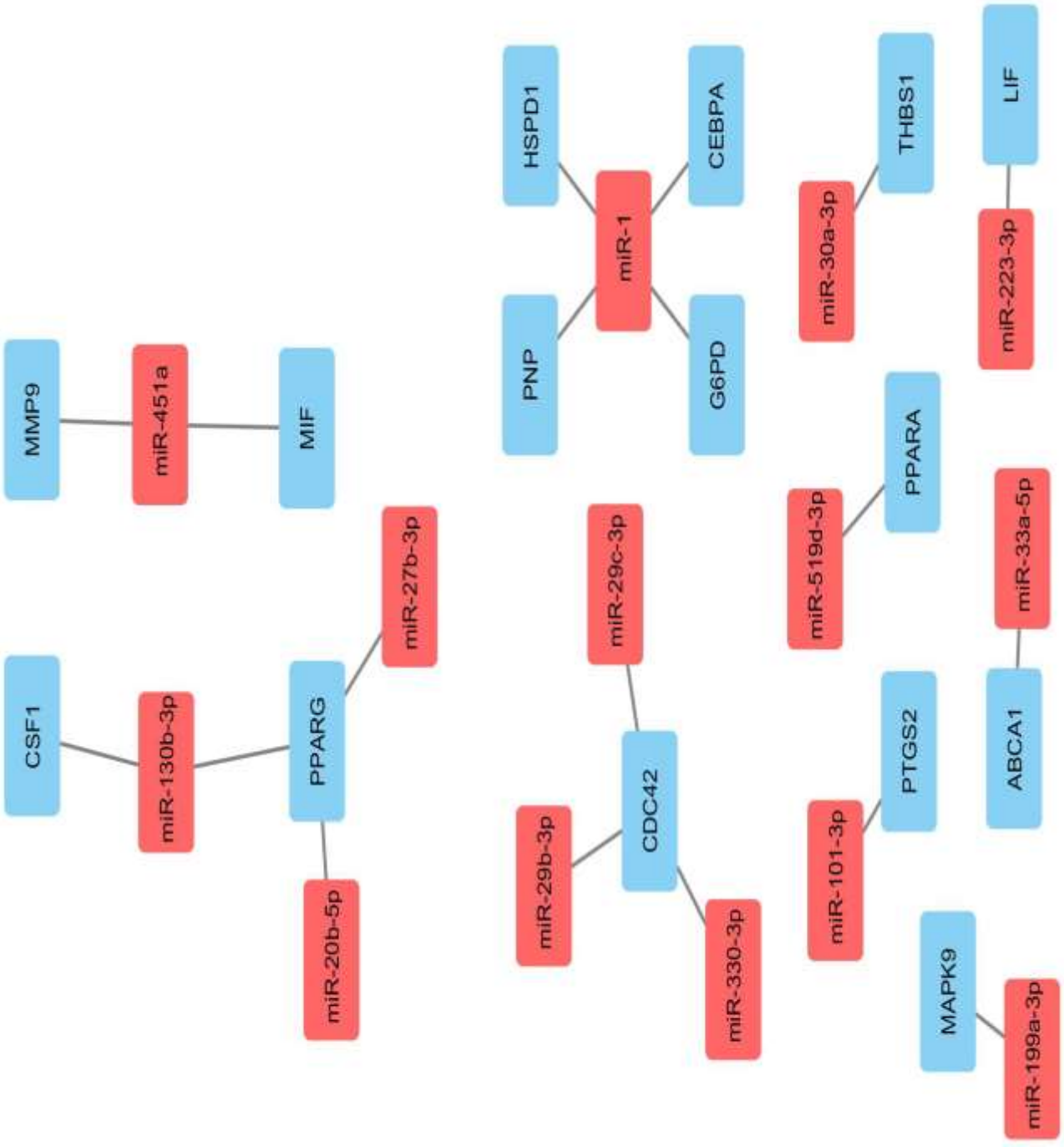


Figure 4.9 microRNAs experimentally validated to target genes involved in the regulation of Macrophages Activation. Integrative target prediction analysis by Cytoscape using DE- miRNAs among comparison. In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

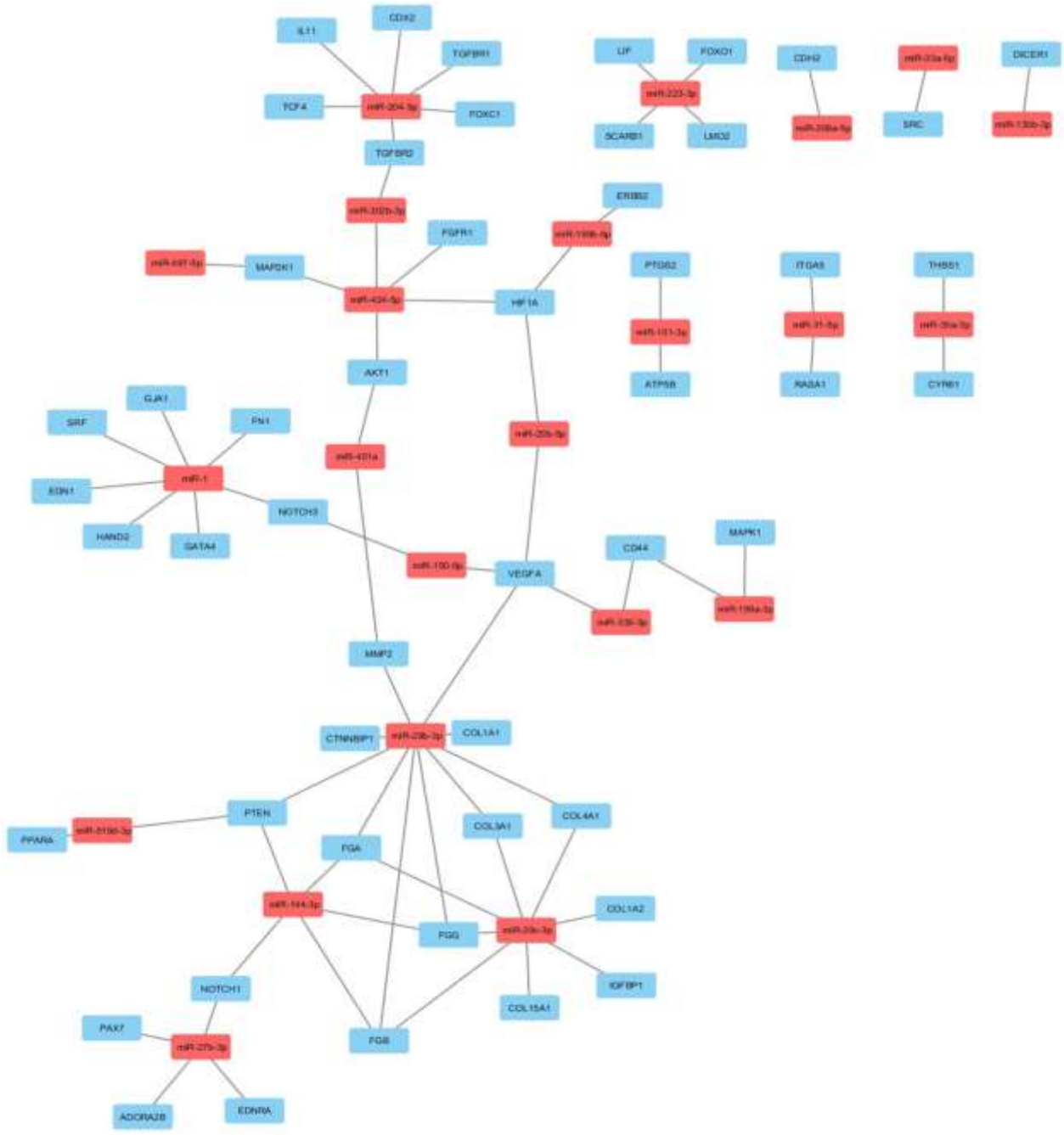


Figure 4.10 microRNAs experimentally validated to target genes involved in the regulation of Endothelial cells activation and differentiation. Integrative target prediction analysis by Cytoscape using DE- miRNAs among comparison. In the red boxes are reported microRNAs de-regulated in the comparison between rejections, in the blue boxes the validated target genes.

MicroRNAs that were differentially expressed in almost two comparisons between rejections types and that had been validated to target genes involved in

pathways peculiar of rejection were selected as possible biomarkers. The complete list of the microRNAS is depicted in the table 4.1.

Table 4.2 List of selected microRNAs as possible biomarkers.

miRNA	mirTarBase	Entrez Id	miRNA	mirTarBase	Entrez Id
hsa-miR-126-5p	MIRT001820	85414	**hsa-miR-29b-3p**	MIRT000096	9759
	MIRT005728	5778		MIRT000097	56998
	MIRT007267	8754		MIRT000098	50509
	MIRT007268	4316		MIRT000100	1277
	MIRT007380	6387		MIRT000445	6667
hsa-miR-199a-3p	MIRT001969	4233		MIRT000684	1021
	MIRT006560	6595		MIRT000930	23621
	MIRT003790	960		MIRT006486	23368
	MIRT003982	5601		MIRT002310	6421
	MIRT003983	5594		MIRT002316	51726
	MIRT006453	2526		MIRT006251	4678
	MIRT006655	858		MIRT006098	5728
hsa-miR-27b-3p	MIRT006547	904		MIRT003026	1789
	MIRT001768	4851		MIRT003029	1788
	MIRT006522	5077		MIRT003287	4170
	MIRT006510	1576		MIRT003290	596
	MIRT003388	6768		MIRT003661	1786
	MIRT003427	4322		MIRT003736	6285
	MIRT004499	136		MIRT003813	7422
	MIRT004826	1545		MIRT004308	2099
	MIRT004863	10597		MIRT004312	8202
	MIRT005023	5468		MIRT004419	80312
	MIRT005753	1909		MIRT004510	8115
	MIRT005754	2070		MIRT006488	998
	MIRT006509	7421		MIRT005383	4324
	MIRT006988	8570		MIRT005385	10893
	MIRT023463	5081		MIRT005486	2896
hsa-miR-451a	MIRT000046	4282		MIRT005522	2266
	MIRT000500	51719		MIRT005533	2243
	MIRT006358	4609		MIRT005534	2244
	MIRT006533	51552		MIRT005567	1281
	MIRT005656	5243		MIRT005568	1282
	MIRT005740	207	**hsa-miR-29c-3p**	MIRT006386	3484
	MIRT005742	4313		MIRT006384	7022

	MIRT005743	4318		MIRT006383	81578
	MIRT005744	596		MIRT000927	1281
	MIRT006597	51014		MIRT000928	1277
hsa-miR-144-3p	MIRT003058	5324		MIRT006489	998
	MIRT005523	2266		MIRT001921	6996
	MIRT005529	2243		MIRT001922	6678
	MIRT005530	2244		MIRT001923	3915
	MIRT005869	4851		MIRT001925	1306
	MIRT006872	2475		MIRT001926	1284
	MIRT007190	5728		MIRT001927	1282
	MIRT007310	4780		MIRT001928	1278
miRNA	**mirTarBase**	**Entrez Id**		**mirTarBase**	**Entrez Id**
hsa-miR-190a-5p	MIRT005066	1027		MIRT001929	2597
	MIRT021082	23239		MIRT006487	23368
hsa-miR-218-5p	MIRT001057	10150		MIRT003025	1789
	MIRT001058	1942		MIRT003028	1788
	MIRT001059	9688		MIRT003200	1021
	MIRT001060	23107		MIRT003288	4170
	MIRT001061	10682		MIRT003289	596
	MIRT001062	3914	**hsa-miR-208a-5p**	MIRT005526	2244
	MIRT006105	253260		MIRT005527	2243
hsa-miR-31-5p	MIRT006468	55662		MIRT006385	54361
	MIRT000088	387		MIRT006382	1294
	MIRT000490	5520		MIRT020415	4613
	MIRT000491	26524		MIRT005066	1027
	MIRT006567	23314		MIRT021082	23239
	MIRT001180	50943		MIRT000586	1026
	MIRT004019	6401	**hsa-miR-135a-5p**	MIRT000630	5465
	MIRT004597	846		MIRT006198	7077
	MIRT004968	7528		MIRT006196	5728
	MIRT004969	5979		MIRT006197	10000
	MIRT004970	8650		MIRT000707	3717
	MIRT004971	10725		MIRT001167	4306
	MIRT004972	51621		MIRT007105	4609
hsa-miR-302b-3p	MIRT003555	894			
	MIRT005670	648			
	MIRT005930	7048			
	MIRT005933	389			
	MIRT007172	207			

4.5 Confirmation with qRT-PCR of selected microRNAs identified from NGS analysis.

After these results on the study population I enrolled other EMBs from 13 pts selected according to our inclusion and exclusion criteria in order to validate the NGS results in a greater population.

I performed on all the 33 EMbs (both study and validation cohorts) qRT-PCR of the microRNAs selected. The relative expression levels were normalized to the reference gene (RNU46) and logarithmically transformed (log10) (Qbase, Biogazelle). Overall, the qRT-PCR data showed a similar trend in miRNAs expression as the one revealed by NGS analysis. Taken together these results show a modulated, deregulated expression of a series of miRNAs clusters playing important roles in heart transplant rejection.

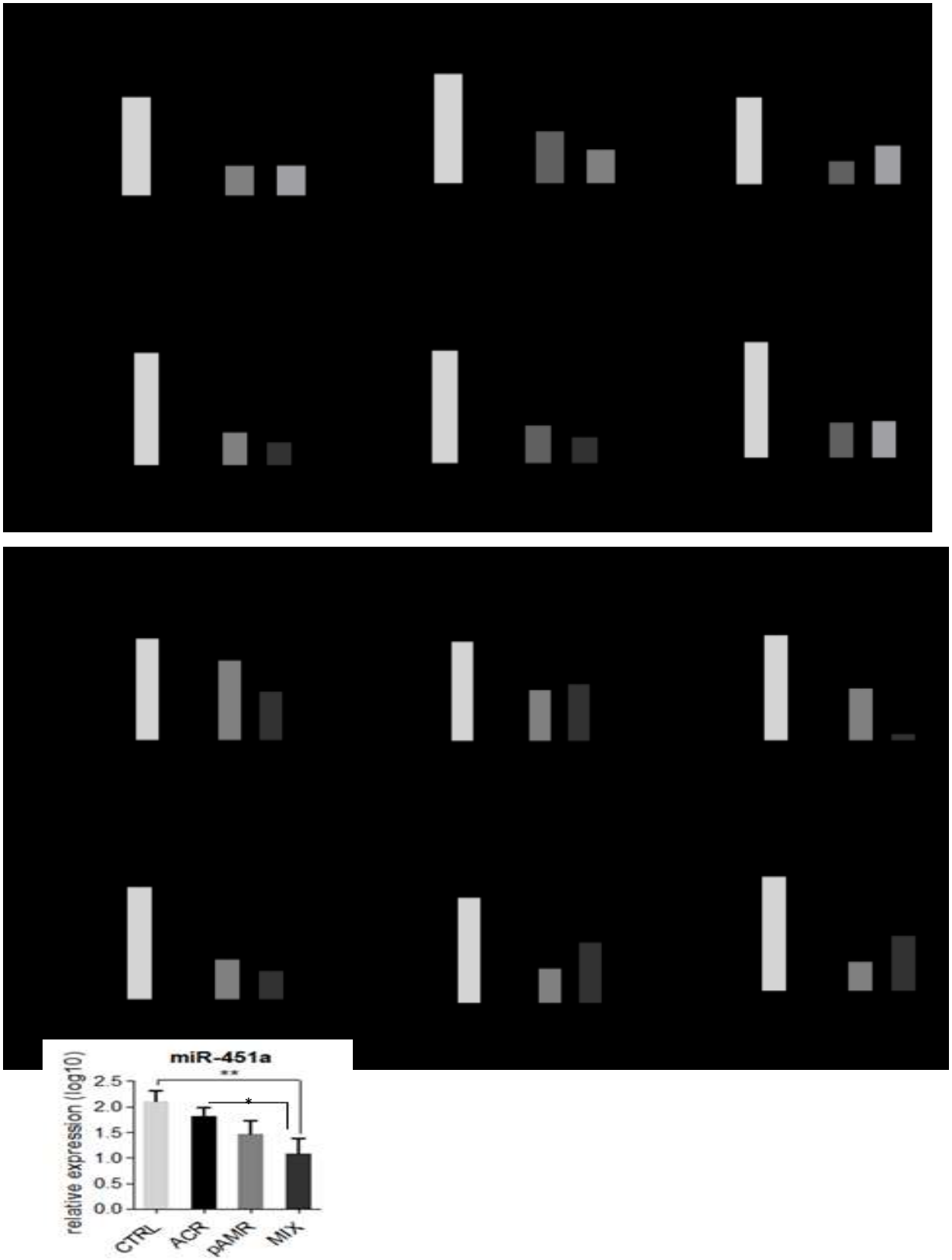


Figure 4.11 Relative expression of selected microRNAs in the 3 rejections and control group. The expression levels of the microRNAs were quantified in FFPE-EMBs . *P < 0.05 and ***P < 0.001. P values were obtained by Student's t test.

Those miRNAs I chose, have a good discrimination strength for each type of rejection in particular: miR-218-5p differentiate both AMR and MIX from ACR; miR-31-5p, miR-29b-3p, miR-29c-3p differentiate ACR from AMR, miR-208a-5p, miR-27b-3p and miR-451a differentiate ACR from MIX while miR-190-5p, miR-135a-5p, miR-144-3p, miR-126-5p and miR-199a-5p differentiate ACR from Mix and AMR.

4.6 ROC analysis of the selected microRNAs and creation of Logistic Regression model predictive in future applications

To confirm that miRNAs selected are able to expressly identify only the rejection between control and pathological biopsies, I conducted sensitivity analysis. I stratified the ROC analysis by rejection diagnosis and found that 11 miRNAs are biomarker of rejection. In detail: miR-27b-3p (AUC= 0.937), miR-29b-3p (AUC=0.942) miR-29c-3p (AUC=0.852) miR-126-5p (AUC=0.825) miR-135-5p (AUC=0.868) miR-144-3p (AUC=0.841) miR-190-5p(AUC=0. 873) miR-199a-3p(AUC=0.937) miR-223-3p (AUC=0.841) miR-302b-3p (AUC= 0.884) miR-451a (AUC=0.783).

Because the levels of each single miRNA cannot recognize a type of rejection in a unique way, a next step in my experimental approach was to integrate the data in unique miRNA signatures that could be used as a predictive model in future applications. I included the expression data of miRNAs showing significant discriminative power for rejection (Fig 4.12) and/or miRNAs differentially expressed in a single condition (vs the other groups) (Fig 4.13) as co-variates in logistic regression models aimed to statistically identify a

specific rejection type. The first model with miR-208, miR-126-5p, miR-135-5p as covariables, considering a cutoff value of 0.5, conferred respectively 90.0% discrimination between MIX vs others, 100.0% discrimination between MIX vs ACR and 80.0% discrimination between MIX vs pAMR. . The second regression model with miRNAs -27b,-29b,-199a-3p, -208a, 302 as covariables b is able to correctely predict 76.7%, 80.0%, 64.7% of ACR vs other, ACR vs. pAMR and ACR vs MIX respectively. The third model composed by the variables miRNAs-208-,29b-3p,- 135 and -144-3p correctly predict 90.0% of pAMR vs others, 100% of pAMR vs ACR and 70% of pAMR vs MIX. The receiver operating characteristic curve for the logistic regression models with table reporting the overall percentage are shown in Figure 4.13.

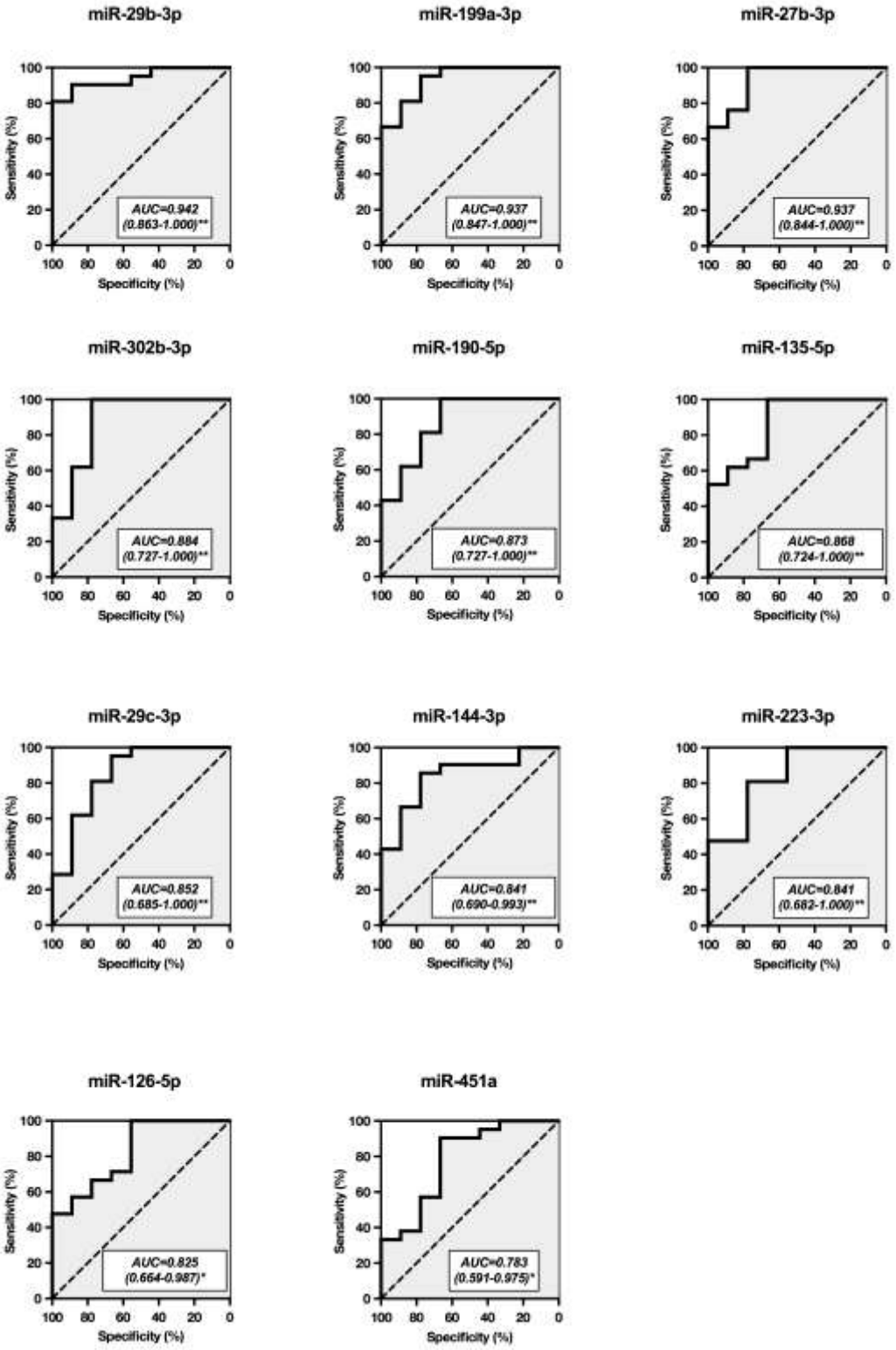


Figure 4.12 ROC curve of miRNAs to predicted rejection and non-rejection. The sensitivity and specificity of miR-27b-3p (AUC= 0.937), miR-29b-3p (AUC=0.942) miR-29c-3p (AUC=0.852) miR-126-5p (AUC=0.825) miR-135-5p (AUC=0.868) miR-144-3p (AUC=0.841) miR-190-5p(AUC=0. 873) miR-199a-3p(AUC=0.937) miR-223-3p (AUC=0.841) miR-302b-3p (AUC= 0.884) miR-451a (AUC=0.783)

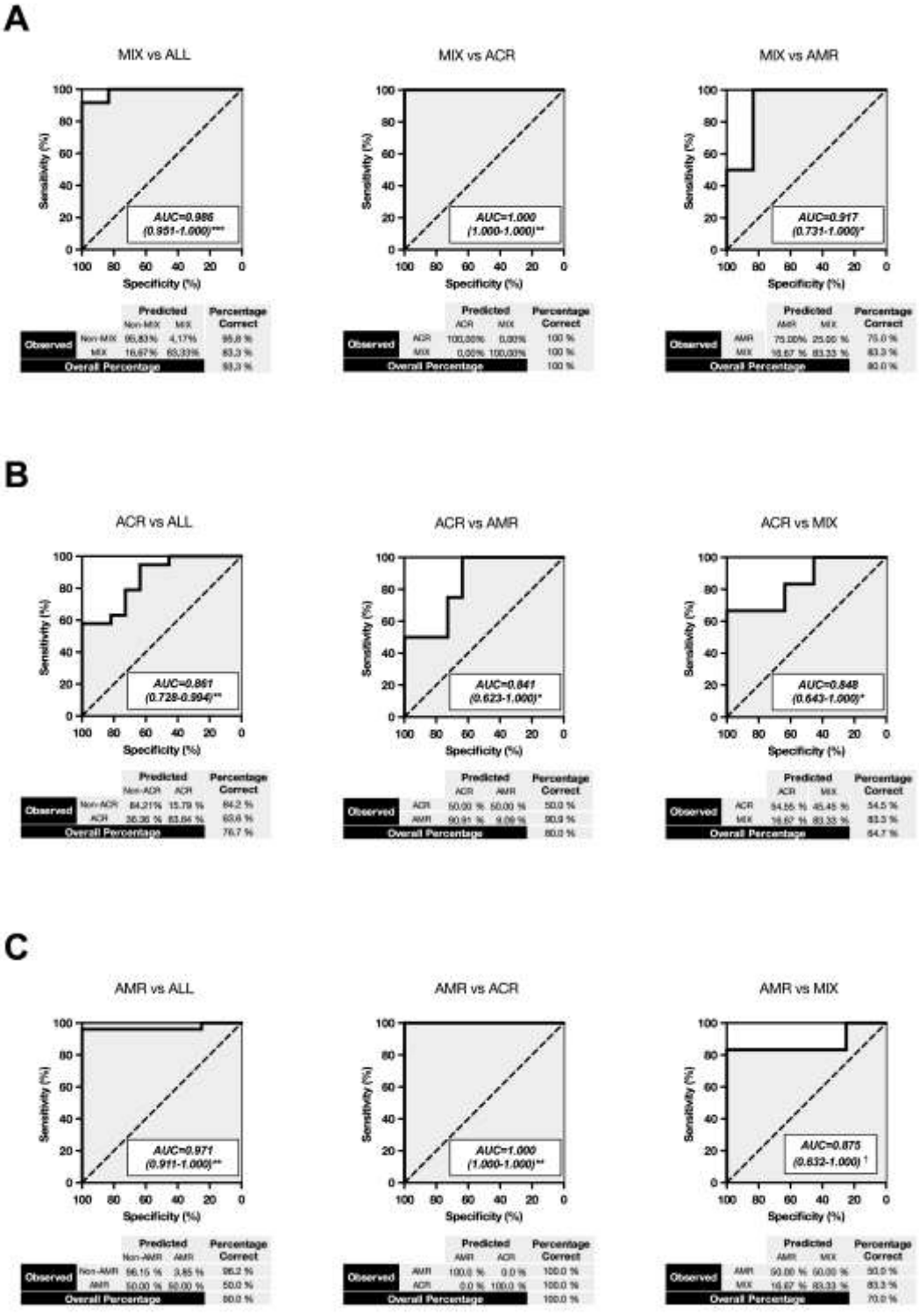


Figure 4.13 Receiver-operating characteristic curve derived from the logistic regression models. (A) Model developed on miR-208, miR-126-5p, miR-135-5p as covariables conferred respectively 90.0% discrimination between MIX vs others, 100.0% discrimination between MIX vs ACR and 80.0% discrimination between MIX vs pAMR . (B) Model developed on miRNAs -27b,-29b,-199a-3p, -208a, 302 as covariables conferred 76.7% discrimination between ACR vs other, 80.0% ACR vs. pAMR , 64.7%, ACR vs MIX respectively. (C) Model developed on miRNAs-208-,29b-3p,- 135 and -144-3p correctly predict 90.0% of pAMR vs others, 100% of pAMR vs ACR and 70% of pAMR vs MIX.

4.7 In situ PCR.

Among the 13 microRNAs we started with 4 miRNAs that had higher expression in the NGS experiment: I selected: miR-29b-3p, miR-144-3p, miR-126-5p and miR451a. I selected from ACR and pAMR group two serial sections one of which was used for negative control staining. After deparaffinization and DNase digestion we performed In situ PCR. This method involves the extension of the labelled miRNA hybridized to a template with 100-nucleotide–long ultramer that contains the complementary sequence of the miRNA 3′-UTR. The extension method results in visualizing the miRNA signal in specific cells and tissues with using RT-PCR. Sections were subsequently stained with antibodies specific for a cell type followed by fluorophore-conjugated secondary antibodies

4.7.1 Expression of miR-29b-3p in ACR and AMR.

In the figure 4.13 the H&E staining showed the acute cellular rejection with multifocal cardiomyocytes damage with monocytes infiltrate. In pAMR the inflammatory burden was present into the capillaries without cardiomyocyte damage and focal interstitial oedema. miR-29b-3p co-localized with fibroblast or smooth muscles cells of arteriolar parietal wall (SMA Positive cells) (fig.4.14) in ACR. The staining for cardiomyocytes (Troponin positive cells) and macrophages (CD68 positive cells) did not show important co-localization with miR29b-3p. In AMR cases (pAMR positive and circulating DSA+) miR29-3p co-localized with cardiomyocytes, on the contrary smooth muscle cells and macrophages did not show the co-localization - Furthermore the

expression of the microR29b-3p was different in the two types of rejection, more intense and diffuse in the ACR samples compared to the pAMR

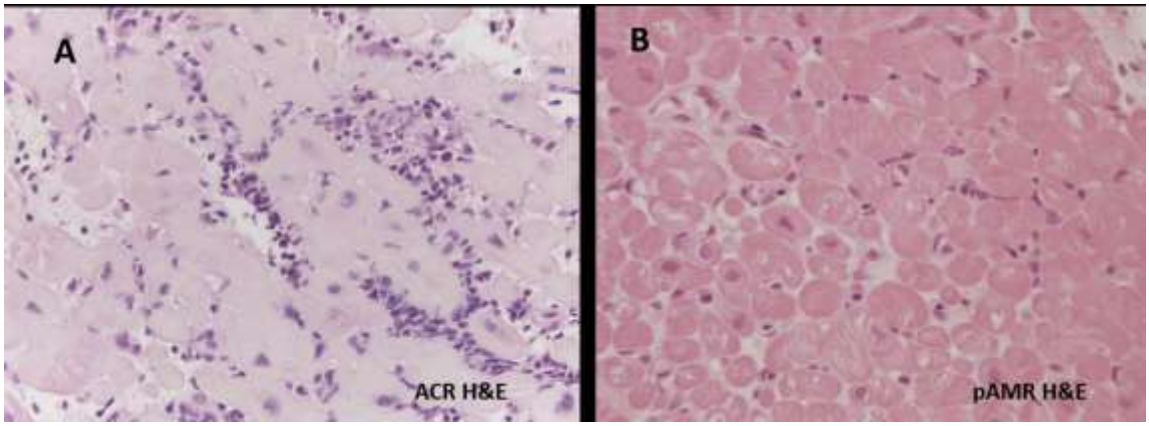


Figure 4.13 Histopathological characteristics of human endomyocardial biopsy. A) Acute cellular H&E staininig and B) Antibody mediated rejection H&E stained

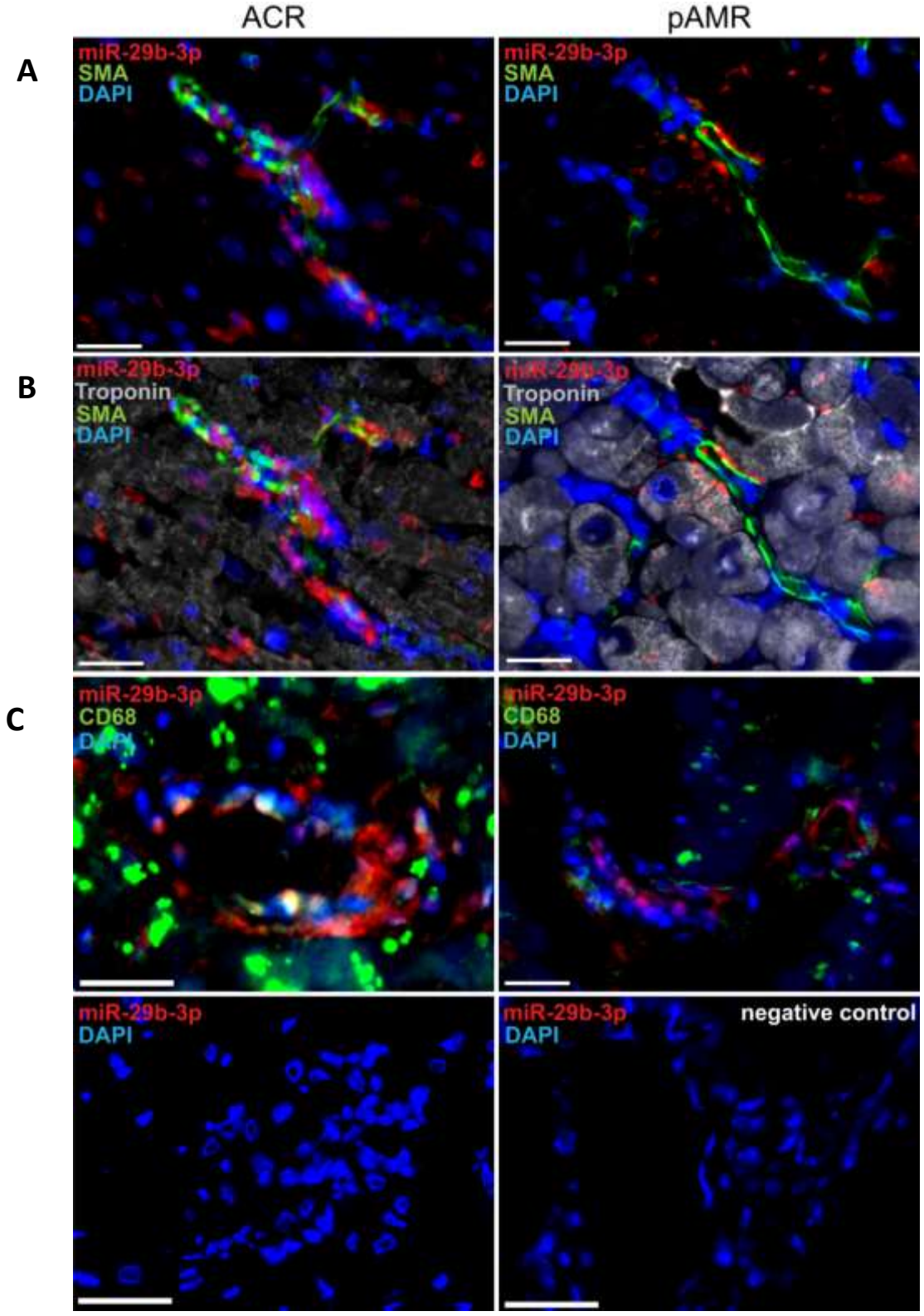


Figure 4.14 MiR-29b-3p expression in human endomyocardial biopsies. Localization of miR-29b-3p in human FFPE-EMBs was determined by *in situ* PCR for miR-29b-3p combined with immunostaining for SMA (A) , SMA and Troponin(B) and CD68 (C) in ACR and AMR (representative images were shown). Nuclei were counterstained with DAPI. Scale bars, 25 µm. 40X magnification.

4.7.2 Expression of miR-144-3p in ACR and AMR

In the figure 4.5.2 in situ PCR showed the expression of miR144 in endothelial cells (von Willebrand factor positive cells) in ACR group. In pAMR group miR144-3p co-localized properly with lymphocytes (CD3 positive cells).

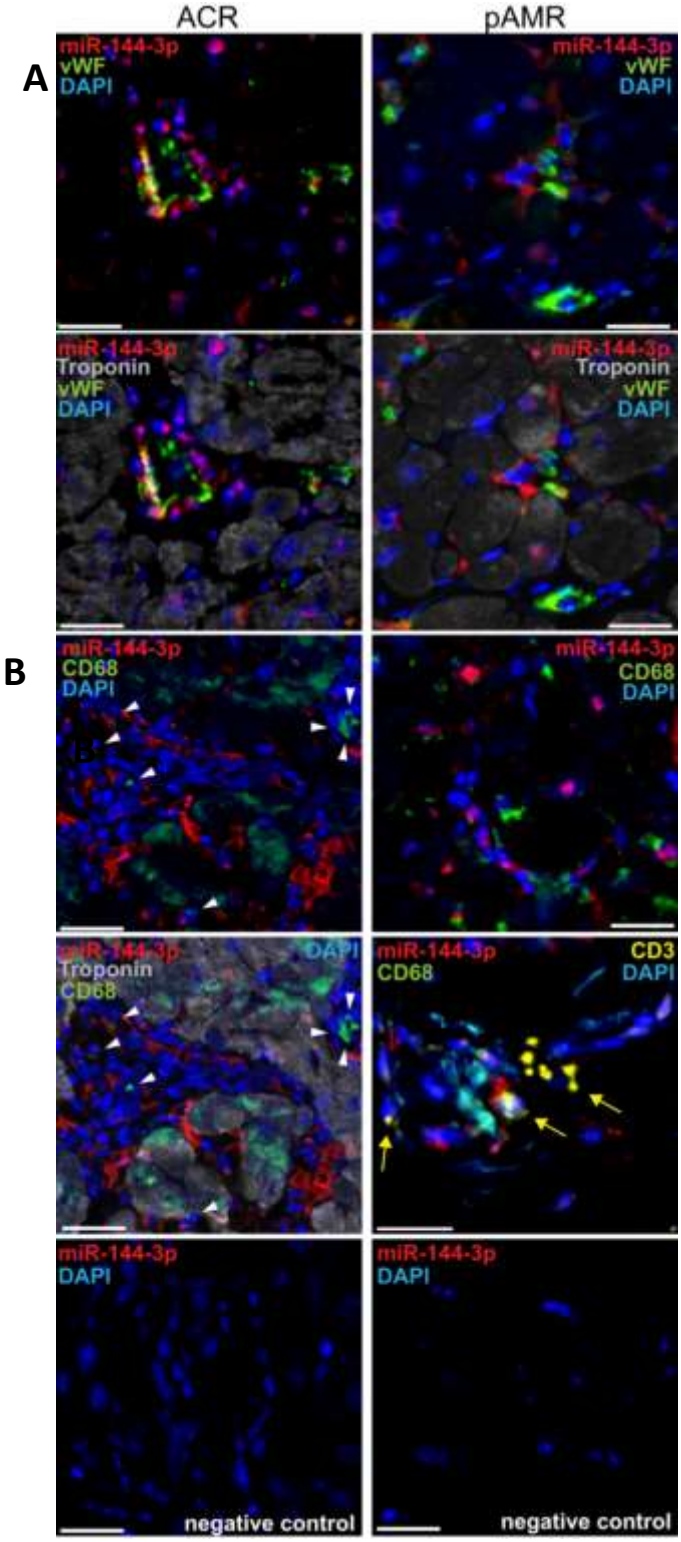


Figure 4.15 MiR-144-3p expression in human endomyocardial biopsies. Localization of miR-144-3p in human FFPE-EMBs was determined by in situ PCR for miR-144-3p combined with immunostaining for vWF and Troponin (A) and CD68 and Troponin (B) in ACR and AMR (representative images were shown). Nuclei were counterstained with DAPI. Scale bars, 25 μm. 40X magnification

4.7.3 Expression of miR-126-5p in ACR and AMR

The figure 4.15 shows the co-localization of the miR-126-5p in ACR and pAMR samples in endothelial cells, on the contrary in pAMR group the same miRNA was express in cardiomyocytes.

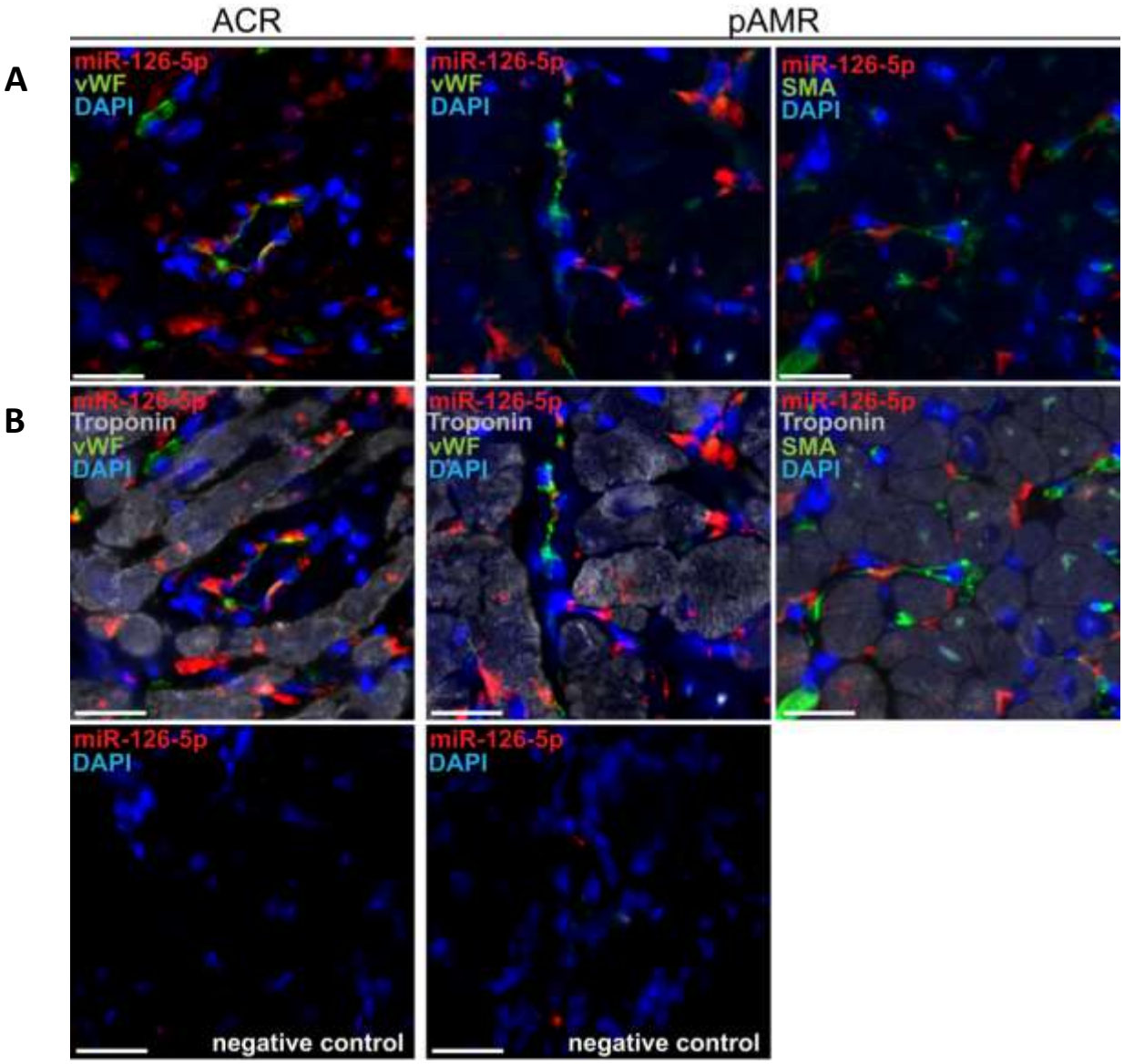


Figure 4.16 MiR-126-5p expression in human endomyocardial biopsies. Localization of miR-126-5p in human FFPE-EMBs was determined by in situ PCR for miR-126-5p combined with immunostaining for vWF and Troponin in ACR and AMR (A), SMA and SMA and Troponin in AMR (B) (representative images were shown). Nuclei were counterstained with DAPI. Scale bars, 25 μm. 40X magnification

4.7.4 Expression of miR-451a in ACR and AMR

In the figure 4.17 we tested the expression of miR-451a in smooth muscle/ fibroblast cells (SMA positives) and cardiomyocytes (Troponin positive cells) in ACR sample and in endothelial cells (vWF positive) and cardiomyocytes (troponin positive cells) in AMR. In the ACR group there was positive co-localization in smooth muscle cells (SMA positive cells) and in lymphocytes (CD3+). On the contrary in the pAMR group the co-localization was present in endothelial cells (von Winlembrand positive cells) . There was a different expression of miR-451a in the two types of rejection ACR and pAMR.

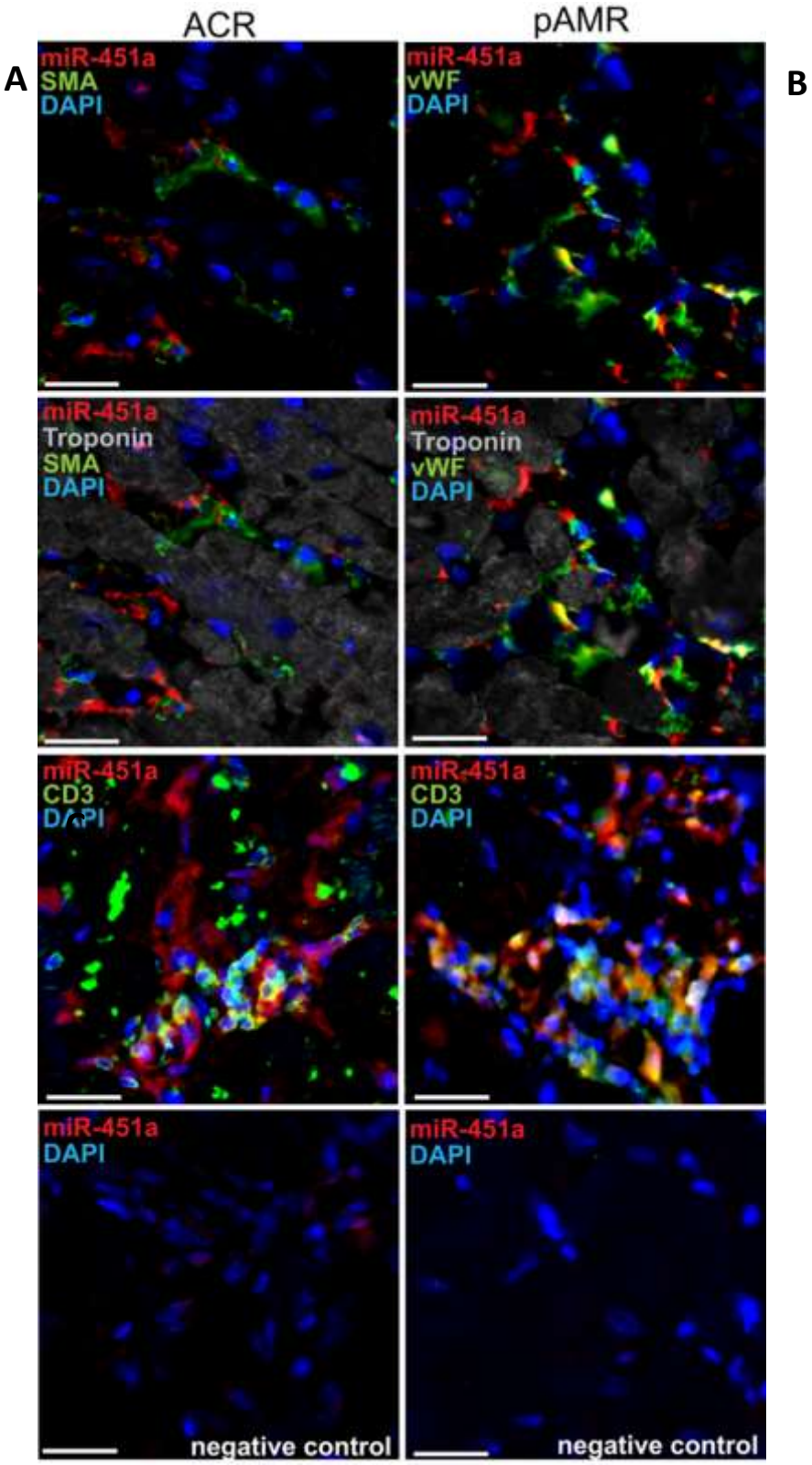


Figure 4.17 MiR-451a expression in human endomyocardial biopsies. Localization of miR-451a in human FFPE-EMBs was determined by in situ PCR for miR-451a combined with immunostaining for SMA in ACR(A) and vWF in AMR (B); for CD3 in ACR and AMR (C) (representative images were shown). Nuclei were counterstained with DAPI. Scale bars, 25 μm. 40X magnification

5 Discussion.

MicroRNAs are small non-coding RNAs of 20-22 nucleotides involved in transcriptional and post-transcriptional regulation of gene expression. MiRNAs function via base-pairing with complementary sequences within target mRNA molecules, usually resulting in gene silencing via translational repression or target degradation. MiRNAs are involved in several physiological functions, and several studies have demonstrated abnormal expression levels of miRNAs in different pathologies. Biomarkers from easy accessible compartments, such as peripheral blood, plasma, serum, urine, saliva, and cerebrospinal fluid could be used to detect and monitor the disease much earlier, even before symptoms appear. For my PhD thesis I decided to consider FFPE tissues as potential diagnostic sources. Formalin fixation and paraffin embedding for the conservation of tissue samples has been applied for over 100 years in all areas of biomedical research and diagnostics. Hence, archives with formalin-fixed and paraffin-embedded (FFPE) diagnostic tissue samples with associated clinical information or samples from elaborate and costly scientific experiments of tremendous value have accumulated worldwide. These precious biologic samples have commonly been used to answer the scientific or diagnostic questions by application of histologic techniques to visualize morphologic and immunohistochemical features associated with certain pathologic or physiologic conditions. With the discovery of DNA, RNA and, recently, small interfering RNA like miRNA as well as associated amplification and visualization techniques the possibilities for characterizing diseases have expanded far beyond the level of morphology. The goal of my

study was to identify clusters of miRNAs that characterize with high sensitivity and specificity the different types of rejection in heart transplanted patients. Even if DNA and mRNA are reported to be modified by formalin fixation in the contrast the quality of microRNAs (miRNA) seems to be less affected. (99) I applied the NGS technology for the first time in FFPE endomyocardial biopsies routinely used in the monitoring of rejection. The identification of the best method of extraction for short non coding RNAs in FFPE EMBs was the first result I achieved. I tested different methods in house and commercial available kits and I modified the protocols to obtain good quality and adeguate quantity of RNA from FFPE tissue of small EMBs for the downstream application.

5.1 Choice of the best method of extraction

The first goal of my project was the evaluation of the best extraction method of miRNAs from formalin fixed paraffin embedded endomyocardial biopsies, since to date, few studies have been performed on this biological sample, as opposed to frozen biopsies or biological fluids, especially serum and plasma, which have been extensively studied in recent years. Efficient recovery of miRNAs is technically difficult due to their low molecular weight and low abundance in small samples such as formalin fixed endomyocardial biopsies. The isolation of miRNAs is a very important step to study their expression and their correct quantification becomes extremely important. To this end, three extraction protocols have been evaluated for maximum recovery of smallRNAs. To date, many protocols have been developed for the extraction of high quality RNA using different kits and reagents both commercial and

experimental ("home-made"). In general, the different methods are based on the use of: "classic" reagents for nucleic acid extraction, such as guanidine isothiocyanate and phenol (TRIzol or TRI Reagent) solutions or silica-based columns. In order to evaluate the best extractive technique to be applied to endomyocardial biopsies, 3 types of extraction were carried out: Trizol, Phenol / chloroform and ammonium acetate and miRNeasy FFPE kit (Qiagen) columns. To verify the success and compare the types of extraction, first, a measuring with NanoDrop ND1000 a very compact spectrophotometer that provide total RNA concentrations was performed . Then with Agilent 2100 bioanalyzer I tested the quality of the RNA. These analyses led us to choose the use of miRNeasy FFPE kit as extraction method.

5.2 NGS analysis on FFPE EMBs

To have a general view of the overall detected and DE-miRNAs in the examined tissues, we performed the analysis by NGS with semiconductor approach, that allows the detection of even small differences among samples, and enables the identification of annotated miRNAs. Nowadays researchers investigated microRNA expression in heart rejection mainly using microarray platforms on murine model (74) or concentrating their efforts on altered expression of human microRNAs in serum (119). In a recent study Thum et al. analysed the role of a single microRNA, in particular miR-21 from murine and human biopsies in the regulation of fibrosis after transplantation (120) Only a previous study by JPDuong-Van Huyen et al (78) has investigated the expression of miRNA in human HTx. They used frozen tissue and sera to

differentiate pts with rejection from those without and identified four miRNAs , with strong correlation between tissue and sera, miR-10a, MiR-31, MiR-92a, miR155.

In my PhD thesis , that extensively investigates the expression pattern of miRNA in FFPE EMBs in human heart transplanted patients the three types of rejection and control groups were compared in pair with the un-supervised analysis showing a typical profile for each group of differentially expressed miRNAs; in particular: Mixed vs AMR: only 2 miRNAs overexpressed in the Mixed group suggesting a similarity between the two types. ACR vs AMR: 18 miRNAs overexpressed and 2 miRNAs under-expressed in the ACR. Mixed vs ACR : 7 miRNAs under expressed and 39 miRNAs over-expressed in the ACR group. The analysis revealed that there are de-regulated microRNAs between the three rejections confirming our hypothesis that microRNAs can characterize the three pathological conditions. The NGS analysis generated a list of differentially expressed miRNAs in tissues representing a valuable framework to better understand the abnormalities of cellular pathways associated with the pathogenesis of rejections, and to identify candidates for therapeutic intervention. The heat map showed a wide range of miRNA down and up regulated in the different group of patients, some of which are still unknown. Compared to microarray platforms, where there is a limited pre-selected set of probes, the advantage of NGS technique is to select all the miRNAs over or under- expressed in EMBs.

5.2 Validation through qRT-PCR of miRNAs selected.

To validate the NGS data through qRT-PCR we enrolled other EMBs from 13 pts selected according to our criteria and we tested on all the 33 EMbs the selected microRNAs.

MiRNAs have been selected for further evaluation and validation, based on the number of reads resulting by NGS, on their highly significant FDR (< 0.05) or fold change, p-value and their involvement in relevant processes related to rejection as shown by a bioinformatic analysis based on validated target genes and reported in public databases such as TarBase (version 6.0) (111) , miRTarBase (112) , miRWalk (113), miRecords (114), DIANA-microT-CDS (115) , miRmap (116), miRDB (117) , TargetScan (118), and miRanda (119). At the end we selected 12 microRNAs.

The results showed the presence of cluster of miRNAs down and up express in the different types of rejection. In particular intra-graft expression of miR-218-5p, miR-144-3p, miR-190-5p, miR-199a-5p, miR-126-5p, miR135a-5p differentiate both AMR and MIX from ACR; while miR-29b-3p, miR-29c-3p and miR-31-5p differentiate ACR from AMR and miR-27b-3p and miR-208a-5p differentiate ACR from MIX .

The miRNA identified in my analysis were specific for myocardial damage (miR-218-5p, miR-144-3p), interstitial remodelling (miR-29), in the inflammatory burden (miR-31), in accordance with previous studies (78). For some of miRNAs such as miR-199 and miR-190 the role in myocardial rejection is not clear yet even if they have been reported to be involved in

cardiac hypertrophy as shown in the study of Feng at al. (121). The miR-451 in a model of xeno-transplantation have been reported to play a crucial role in the formation of intravascular thrombosis. (122). We noted that for the miR-29b-3p there is a discrepancy between the results of the NGS study and the qRT-PCR. Although it is difficult to interpret, we hypothesized that this can be determined by the similarity between the types of rejection MIX and pAMR therefore it will be the subject of further investigations.

For the first time we evaluate a group of pts affected by mixed rejection. Mixed rejection, has been recognized in the 2013 ISHLT classification as a separate entity from ACR and AMR (93, 101) and has been suggested to represent not just the sum of ACR and AMR but a third entity with a different phenotype and evolution and prognosis.

Through molecular signature we wanted to see if this third entity was in real life a separate process requiring specific therapeutic strategies. The results confirmed our hypothesis showing through the ROC curve analysis that miR-208 ,miR-126-5p, miR-135-5p as co-variables conferred more sensitivity and specificity in differentiating the different types of rejection.

With the strict inclusion and exclusion criteria I adopted, I try to excluded the possible impact produced by different immunosuppressive therapy on the microRNA profiling and the possible influence produced by pre-sensitised pts, LVAD and infections. Therefore my effort was to compare very well defined different groups of patients in terms of rejection.

My observations support the hypothesis that intragraft miRNA expression patterns may be used as biomarkers of human heart allograft status, in

particular in cases where histology did not unravel to the evolution of rejection (122) and in which a complementary method to better characterize the rejection types and guide therapy was needed.

5.3 Logistic regression models as predictive tools of different types of rejection.

Since one miRNA can simultaneously target and regulate hundreds of genes and conversely one gene can be under control by multiple miRNAs simultaneously, I proposed 3 different logistic regression models that identify with good specificity and sensitivity the three types of heart rejection, suggesting that the concomitant evaluation of these miRNAs might represent a new powerful tool to identify pathologic status.

As shown in the figure 4.12 all the microRNAs alone were able to distinguish rejections compared to controls but they didn't have enough sensitivity and specificity to identify each type of rejection. In order to overcome this limitation I tried to combine microRNAs to achieve a good sensitivity and specificity. Among the microRNAs implicated in the regressive logistic model there are two miRNAs that deserve some comments because they are involved in at least two models. microRNA-135-5p was a co-variable of the model specific for MIX and AMR suggesting that this two rejection types could be characterized by similar molecular pathways. The miR-208, that have been reported to be involved in the regulation of cardiac damage (123), is a co-variable of all the regressive model confirming the cardiomyocytes damage as a predominant characteristic of all the rejections.

5.4 Expression of microRNA in different cell types of rejection.

Since the results obtained with the NGS and qRT-PCR were very promising from a diagnostic point of view in the last months of my PhD I tried to make a further step towards identifying possible therapeutic targets. As preliminary results through the in situ PCR I detected the subcellular expression of the first 4 microRNAs within tissue.

These microRNAs as already discussed, until now have been reported to be involved mainly in cardiovascular diseases and in particular in the regulation of tissue damage and endothelial remodelling. According to my results I can speculate that during rejection they are expressed not only in inflammatory, smooth muscle and endothelial cells as already demonstrated but also in cardiomyocytes.

The miR-29 family targets many mRNAs that encode proteins involved in fibrosis, including multiple collagen proteins, fibrillins, and elastin (48). In our samples miR29-3p is expressed in smooth muscle cells of vessels and in the fibroblasts of interstitium, in ACR suggesting an active role of fibroblast in the production of miR-29-3p; instead in pAMR this microRNA was mostly expressed in cardiomyocytes while its expression in smooth muscle cells and macrophages was not so relevant.

In a recent study miR-144-3p have been reported as a potent pro-inflammatory regulator (119) and plays also a role, together with miR-451a, in the regulation

of RAC-1, a gene involved in the regulation of oxidative stress signalling (120). In our samples miR-144-3p was expressed in the endothelium (vWF positive cells) in ACR while it was more expressed in Lymphocytes (CD3 positive cells) in pAMR samples. miRNA 126-5p which was reported to be involved in atherosclerosis (57), was expressed in ACR and pAMR samples in endothelial (vWF positive cells) and also in cardiomyocytes (troponin positive cells) suggesting a new and active role of cardiomyocytes in the pathway of both cellular and humoral rejection. MiR-451a as already mentioned was expressed in cluster with miR-144-3p (120) and has been recently involved in the regulation of lymphocytes (121). In my results in the ACR group it showed positive co-localization in smooth muscle cells (SMA positive cells) and in lymphocytes (CD3+). On the contrary in the pAMR group the co-localization was present in endothelial cells (von Winlembrand positive cells).

This finding not only supports that the variation in the expression of these miRNAs is a true reflection of an immune reaction, but it also indicates that they may be used to predict rejection prior to organ damage has occurred. We can confidently conclude that miRNAs are feasible biomarkers of acute heart rejection. Larger studies are needed, where miRNA sera levels are quantified at the time of routine EMBs to calculate cut-off levels and predictive values for these potential biomarkers, in addition to investigate their changes with variation in immune-suppressive treatment.

6 Conclusions

This study demonstrate that MicroRNAs can be obtained easily from FFPE tissues, miRNAs differentially expressed are involved in pathophysiological mechanisms of rejection such as immune system cells cycle regulation and proliferation, inflammatory pathways NFkB mediated and endothelial remodelling. The miRNAs up or down expressed modulate these pathways in a way peculiar for the different types of rejection. The logistic regression models might represent a powerful diagnostic tool. In situ detection of the miRNAs casts new light on the pathophysiological mechanisms of rejection. Moreover the expression of MiRNAs 144-3p, 126-5p, 29b-3p and 451a identified by in situ PCR in endothelial cells, smooth muscle, inflammatory cells and cardiomyocytes are potential pharmacological targets of rejections.

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