Faculdade de Engenharia da Universidade do Porto

MicroRNAs in Kidney-Heart Fibrosis Crosstalk

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Master's Dissertation
MEB – Master in Biomedical Engineering

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Abstract

Chronic kidney disease (CKD) affects 10% of the world's population and it is predicted to become the fifth most prevalent pathology by 2040. This condition is characterized by a progressive inflammatory and fibrotic state that may lead to organ malfunction and culminate in end-stage kidney disease. Among several comorbidities, CKD is a major risk factor for cardiovascular events and an all-cause of cardiovascular disease (CVD), representing, in advanced ages, a 500-fold increase in the chance for cardiovascular-related mortality. Some evidences show an association between CKD and cardiovascular premature ageing and fibrotic processes. Still, the mechanisms underlying CKD-CVD relationship are largely unknown. In recent years, extracellular microRNAs (miRNAs) were found to participate in the relation between associated diseases with common features. In fact, CKD and CVD share many pathological mechanisms and numerous miRNAs have been implicated in both CKD and CVD alone, but the putative role of circulating miRNAs in the communication between the kidney and the heart pathophysiology has yet not been addressed.

In this context, this work aims to evaluate the expression of a selected miRNA panel in pre-dialysis CKD patients for investigating its role as diagnostic biomarkers and therapeutic targets, as well as its association with cardiovascular outcomes.

Results showed that miR-30c-5p and miR-132-3p were significantly downregulated in CKD patients and a receiver operating characteristic (ROC) analysis revealed that a combinatorial panel with both miRNAs allows a better discrimination of the disease with a sensitivity of 83.7% and a specificity of 76.9%. In addition, miR-324-3p was significantly downregulated in CKD patients that presented disease progression in 5 years follow up. Furthermore, the expression of the miRNAs was also evaluated in CKD patients with and without CVD and the results revealed that miR-199a-5p was significantly upregulated in patients with both comorbidities.

In conclusion, miR-30c-5p and miR-132-3p were identified as potential diagnostic biomarkers for early CKD diagnosis and miR-324-3p was identified as a prognostic biomarker for disease progression and also as a putative target for preventing the progression to worse stages. Finally, miR-199a-5p was identified as a putative mediator between kidney and heart fibrotic processes.

Keywords: chronic kidney disease, cardiovascular diseases, microRNAs, fibrosis, biomarkers

Resumo

A doença renal crónica (DCR) afeta 10% da população mundial e prevê-se que se torne na quinta patologia mais prevalente até 2040. Esta doença é caracterizada por um estado progressivo de inflamação e de fibrose que leva à falência do órgão e culmina numa doença renal em estadio terminal. Dentro das várias comorbilidades, a DCR é um grande fator de risco de eventos cardiovasculares e uma grande causa de doenças cardiovasculares (DCV), representando, em idades mais avançadas, uma probabilidade de 500 vezes superior de falecer devido a uma DCV. Algumas evidencias mostram uma associação entre a DCR e os prematuros processos de envelhecimento e de fibrose cardiovasculares. Mesmo assim, os mecanismos subjacentes à relação DCR-DCV continuam desconhecidos. Nos últimos anos, descobriu-se que os miRNAs extracelulares participam na relação de doenças associadas com características comuns. De facto, a DCR e a DCV partilham mecanismos patológicos e vários miRNAs têm vindo a ser implicados tanto na DCR como na DCV, mas o suposto papel dos miRNAs circulantes na comunicação entre a fisiopatologia do rim e do coração ainda não foi abordado.

Neste contexto, este trabalho tem como objetivo avaliar a expressão de um painel selecionado de miRNAs em pacientes com DCR em pré-diálise para investigar o seu papel como biomarcadores de diagnóstico e alvos terapêuticos, assim como a sua associação com eventos cardiovasculares.

Os resultados mostraram que a expressão do miR-30c-5p e do miR-132-3p estava significativamente diminuída em pacientes com DCR e a análise ROC revelou que um painel combinatório com ambos os miRNAs permite uma melhor discriminação da doença com uma sensibilidade de 83.7% e uma especificidade de 76.9%. Para além disso, os resultados mostraram que a expressão do miR-324-3p estava significativamente diminuída em pacientes com progressão de estadio. Ainda, a expressão dos miRNAs também foi avaliada em pacientes com DCR que sofriam ou não de uma DCV e os resultados revelaram que a expressão do miR-199a-5p estava significativamente aumentada em pacientes com as duas comorbilidades.

Para concluir, o miR-30c-5p e o miR-132-3p foram identificados como possíveis biomarcadores de diagnóstico para a deteção precoce da DCR e o miR-324-3p foi identificado como biomarcador de prognóstico de progressão de doença, assim como sendo um possível alvo para prevenir a progressão para estadios mais avançados. Por fim, o miR-199a-5p foi identificado como um possível mediador entre os processos de fibrose renal e cardíaca.

Palavras-chave: doença renal crónica, doenças cardiovasculares, microRNAs, fibrose, biomarcadores

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Abbreviations

List of Abbreviations:

A

ACE	Angiotensin-Converting Enzyme
ACEi	Angiotensin-Converting Enzyme inhibitor
ACR	Albumin-to-Creatinine Ratio
Ago	Argonaute
AKT	Protein Kinase B
Ang-II	Angiotensin-II
APD	Automated Peritoneal Dialysis
AUC	Area Under the Curve

B

BLI	Bioluminescence Imaging

C

CAPD	Continuous Ambulatory Peritoneal Dialysis
cDNA	Complementary deoxyribonucleic acid
CFs	Cardiac Fibroblasts
CHUSJ	Centro Hospitalar Universitário de São João
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease – Epidemiology Collaboration
CRP	C-Reactive Protein
CTGF	Connective Tissue Growth Factor
CVD	Cardiovascular Disease

D

DCM	Dilated Cardiomyopathy
DCR	Doença Crónica Renal
DCV	Doença Cardiovascular
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DN	Diabetic Nephropathy
DNA	Deoxyribonucleic Acid

E

ECM	Extracellular Matrix

eGFR	Estimated Glomerular Filtration Rate
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinases

F

FEUP	Faculdade de Engenharia da Universidade do Porto
FGF23	Fibroblast Growth Factor 23
FMUP	Faculty of Medicine of Porto University

G

GFR	Glomerular Filtration Rate

H

HD	Healthy Blood Donors
HDL-C	High-Density Lipoprotein Cholesterol
HG	High Glucose
HIV	Human Immunodeficiency Virus
HK	Human Kidney

I

i3S	Instituto de Investigação e Inovação em Saúde
IgA	Immunoglobulin A
IJUP	Investigação Jovem da Universidade do Porto
IL-1β	Interleukin-1β
IL-6	Interleukin-6

K

KD	Knockdown
KDIGO	Kidney Disease Improving Global Outcomes
KO	Knockout

L

LAD	Left Anterior Descending Coronary Artery
LDL-C	Low-Density Lipoprotein Cholesterol
LPS	Lipopolysaccharide
LV	Lentivirus

M

MAPK	Mitogen Activated Protein Kinase
Max	Maximum
MCP-1	Monocyte Chemoattractant Protein-1
MEFs	Mouse Embryonic Fibroblasts
MI	Myocardial Infarction
Min	Minimum
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MiRNAs	MicroRNAs
MMP-2	Matrix Metalloproteinase-2
mRNA	Messenger RNA

N

NFAT	Nuclear Factor of Activated T-cells
NF-κB	Nuclear Factor Kappa B
NIRD&DG	Nephrology and Infectious Diseases Research and Development Group

O

OLETS	Otsuka-Long-Evans-Tokushima-Fatty

P

PAMAMs	Poly(Aminodoamine)
PD	Peritoneal Dialysis
PEI	Polyethyleneimine
PI3K	Phosphoinositide 3-Kinases
PLGA	Poly(Lactile-co-Glycolic Acid)
pmp	Per Million Population
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA

Q

qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction

R

RAAS	Renin-Angiotensin-Aldosterone System
rAAV	Recombinant Adeno-Associated Virus
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA interference
ROC	Receiver Operating Curve
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy

S

SCr	Serum Creatinine
SCysC	Serum Cystatin C
SD	Sprague Dawley
SEM	Standard Error of the Mean
shRNA	small hairpin RNA
Smad	Small Mothers Against Decapentaplegic
SNS	Sympathetic Nervous System
SPN	Sociedade Portuguesa de Nefrologia
STZ	Streptozotocin

T

TAC	Transverse Aortic Constriction
TECs	Tubular Epithelial Cells
TGF-β	Transforming Growth Factor Beta
TGF-β1	Transforming Growth Factor Beta 1
TNF-α	Tumor Necrosis Factor Alfa
TRBP	Transactivation Response RNA-binding Protein

U

UTR	Untranslated Region
UUO	Unilateral Ureteral Obstruction

W

Wnt	Wingless Integration Site
WT	Wild-Type

List of Symbols:

ρ	Spearman's Rho coefficient

Chapter 1

Introduction

1.1 - Context

Chronic kidney disease (CKD) is a highly debilitating illness defined by a progressive loss of kidney function and structure alterations that may, ultimately, result in organ failure and the need for renal replacement therapies (RRT), such as dialysis or kidney transplant [1]. Indeed, CKD represents a strong risk factor for cardiovascular diseases (CVD), increasing the cardiovascular risk up to 500-fold. On the other hand, CVD is the main cause of death amongst CKD patients, being responsible for 50% of them [2]. This suggests the existence of a bidirectional relationship between the kidney and the heart, but the pathophysiological pathways involved in the intercommunication between these two organs are still far from being known. Fibrosis is one of the common events that leads to organ failure in both diseases, and it has been described that small regulatory molecules called microRNAs (miRNAs) are involved in this process [3]. In this sense, miRNAs act at a post-transcriptional level regulating gene expression and can circulate in the bloodstream, so they could play a key role in the kidney-heart crosstalk during CKD [4]. Considering the enrollment of miRNAs in both the kidney and heart fibrosis and the fact that these molecules are found in circulation, the investigation of its putative role in kidney-heart crosstalk may not only elucidate on the underlying mechanisms but also i) provide new biomarkers for early CKD diagnosis and CVD risk assessment and ii) new therapeutic targets for disease management.

This project was developed as part of the curricular unit Dissertação of the Master degree in Biomedical Engineering at Faculty of Engineering of Porto University (FEUP) and it was carried out at the Nephrology and Infectious Diseases Research and Development Group (NIRD&DG) of Instituto de Investigação e Inovação em Saúde (i3S) in close collaboration with the Department of Nephrology of Centro Hospitalar Universitário de São João (CHUSJ) and Faculty of Medicine of Porto University (FMUP). Moreover, this research was supported by Sociedade Portuguesa de Nefrologia (SPN2020).

1.2 - Motivation

Since I was a little child, my grandfather suffers from heart failure and even had to have cardiac surgery. Now, his condition has worsened and consequently, his kidneys and lungs are starting to get affected, aggravating his quality of life, and making impossible for him to do daily things on his own without depending on someone. Besides, the cardiovascular system has always fascinated me in some way due to its enormous impact on our lives and how easy it can affect the other organs, debilitating us from continuing with our normal lives.

When I read this proposal, besides allowing me to study the CKD, I knew it was my opportunity to enhance my knowledge about the cardiovascular system and its impact on the renal system by investigating the bidirectional relationship between them. Furthermore, it would allow me to study several physiological and pathophysiological mechanisms and learn about miRNAs and their role in this intercommunication.

In the end, my knowledge about the relationship between CKD and CVD will give me a new perspective of the machinery of our body by better understanding the pathophysiological mechanisms involved. In addition, it entails great potential of translation into the clinics throughout the development of new, more specific, biomarkers for early diagnosis of CKD and patient-tailored therapeutic approaches for preventing CKD patients from progressing to worse stages, or to prevent them from developing a cardiovascular condition.

1.3 - Objectives

With this dissertation, both personal and scientific objectives were defined.

From my overall personal and long-term professional perspective, this work is expected to grant me expertise in kidney disease research, namely a better knowledge about CKD and its correlation with the cardiovascular pathology, miRNAs and top of the art research tools, such as real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Moreover, by working with human samples and being in collaboration with the patients and hospital staff, this work will also provide insights into clinical research, such as the need for informed consenting and the difficulties associated with the intrusion into the regular clinical practice.

From a scientific point of view, the proposed research project aims to investigate the expression of a selected panel of circulating miRNAs in pre-dialysis CKD patients at different CKD stages and explore its association with inflammation and interstitial fibrosis in renal biopsies, as well as to investigate their potential as biomarkers and as novel therapeutic targets, looking forward to obtain transferable results to the clinical field.

1.4 - Methodology

For the established aims, I started by performing bibliographic research on the topic of CKD, cardiovascular outcomes and miRNAs in CKD and CVD crosstalk and together with my supervisor, we are currently preparing a revision article on the subject. At the same time, I started to perform laboratory work, learn and perform optimization experiences on plasma separation, miRNA isolation and analysis by real-time qRT-PCR. This period has allowed me to

gain experience in miRNA analysis in human plasma and was described in the curricular unit Projeto de Dissertação that was presented in February 2023.

Following, I started working in the analysis of the miRNA panel in the established cohorts of CKD patients' plasma. Briefly, blood samples were collected from both CKD patients and healthy blood donors (HD), upon signed consent. Then, the plasma was separated by centrifugation, aliquoted and stored at -80°C until further use. Afterwards, ribonucleic acid (RNA) was isolated, reverse transcribed into complementary deoxyribonucleic acid (cDNA) and identified and quantified by real-time qRT-PCR. The miRNAs tested in this study were miR-21-5p, miR-29b-3p, miR-30c-5p, miR-132-3p, miR-146a-5p, miR-199a-5p, miR-203a-3p, miR-324-3p and miR-let-7c-5p. Exogenous miRNAs, cel-miR-39 and cel-miR-54 were used as controls. The obtained results are analyzed using the ΔCq method according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines and statistically analyzed using the GraphPad Prism and SPSS Statistics software.

The work developed until May 2023 was selected for an oral presentation at Investigação Jovem da Universidade do Porto (IJUP).

Throughout this period, I have also been collaborating with sample collection of CKD patients from CHUSJ, performed a brief training in cell cultures and collaborated with two other projects currently ongoing in the NIRD&DG: "Analysis of microRNA expression in peritoneal dialysis patients: its correlation with clinical outputs and microbiome" and "Evaluation of a new player in renal fibroblast activation". This last project was also selected for an oral presentation at IJUP23, and I am one of the authors.

1.5 - Structure

This project is divided into 7 chapters in order to offer a better guidance of the several steps of the work.

Chapter 1 includes the introduction of the theme of the dissertation. In this chapter, the context of the work and personal motivation of the author are described in sections 1.1 and 1.2 respectively, and the respective objectives are identified in section 1.3. In addition, this chapter includes in section 1.4 the proposed methodology to reach the objectives and finishes with section 1.5, presenting the structure of the work.

Chapter 2 presents the literature review. In this chapter, the information about the topic is presented, verified with several cited articles, and organized into three sections. In section 2.1, the overall information about CKD is presented to understand the condition, including generic information about the treatments for CKD, and how inflammation and kidney fibrosis occur in the setting of this disease. In section 2.2, the bidirectional relation between CKD and CVD is presented with a brief explanation about what triggers cardiac fibrosis. In section 2.3, miRNA concepts are presented, including the biogenesis and their role as diagnostic biomarkers and potential therapeutic targets. In addition, it is presented a table showing some miRNAs involved in renal and cardiac fibrosis.

Chapter 3 details the aim of the project as well as the objectives in more specificity.

Chapter 4 includes a detailed methodology of the work performed to achieve the objectives proposed. In sections 4.1 and 4.2, the study population and the sample collection processes are specified. Additionally, sections 4.3, 4.4 and 4.5 describe in detail the RNA isolation, reverse transcription, and qRT-PCR methods, respectively. Finally, section 4.6 presents the softwares and the methods used to analyze the results and to correlate with clinical data.

Chapter 5 presents the results obtained. In this chapter, section 5.1 presents the patient's characteristics that participated in the study. Besides, the results obtained from the real-time qRT-PCR regarding the expression levels of the studied miRNAs are compared between CKD patients and HD, as well as correlated with several clinical data and different comorbidities in the following sections. In addition, section 5.4 presents a table that summarizes the results obtained and in the last section, presents the troubleshooting of the work and how they were overcome.

Chapter 6 includes a detailed and critical discussion regarding the obtained results, which is complemented with information found in the literature in order to establish correlations and to draw some conclusions.

Chapter 7 is the final chapter of the research and presents the general conclusions about the complete development of the work, as well as future work expectations in the area that could be performed to verify the results obtained.

In attachments, a summary of the work I have also performed in collaboration with other colleagues of the research group as well as my attained scientific outputs are presented.

Chapter 2

Literature Review

2.1 - Chronic Kidney Disease

The renal system maintains the fluids, electrolytes and acid-base levels balanced in the organism by keeping a stable exchange between solute and water transport to assure the homeostatic equilibrium of the body, but when the kidneys fail, these life-sustaining functions are compromised affecting other organ systems [5].

According to the Kidney Disease Improving Global Outcomes (KDIGO) (2012), CKD is characterized by the presence of "abnormalities of kidney structure or function, present for > 3 months, with implications for health and classified based on cause, glomerular filtration rate (GFR) category, and albuminuria category" [6]. Nowadays, it represents a major public health problem, being the cause of elevated mortality, morbidity and socioeconomic costs. This condition affects 10% of the world's population [7] and Portugal has one of the highest CKD prevalence in Europe, given that 21% of the Portuguese population suffers from this pathology [8]. In addition, CKD prevalence varies according to gender, age and ethnicity, being higher in women, elderly people and African Americans, respectively [1], [9], [10]. Most importantly, as its prevalence is continuously increasing and there is no cure for this disease, CKD is expected to become the fifth most prevalent cause of death by 2040 [11].

Moreover, CKD entails a huge economic burden that tends to increase at most advanced stages of the disease and with the presence of comorbidities. The European Parliament stated that it is one of the most expensive diseases, costing 140 billion euros annually [12] and in Portugal, the costs suppose a huge expense for public health systems, reaching about 64 million euros in 2019 [13].

Kidney diseases are normally asymptomatic and do not manifest until later stages [6], but patients can exhibit some symptoms including fatigue, pruritus, pain, bloody and foamy urine, nocturia, poor appetite, nausea or dry skin. In more advanced stages, patients may present muscle wasting, asterixis, pallor, pericardial rub and skin excoriations [1], [9], [14].

There are several causes of CKD, but two of the most common are diabetes and hypertension, which are responsible for two thirds of CKD cases [9]. Other causes of CKD development and progression include ageing, obesity, CVD, lupus nephritis, Immunoglobulin A (IgA) nephropathy, prematurity and drug exposure, as well as glomerulonephritis, hepatitis B and C, malaria, tuberculosis and human immunodeficiency virus (HIV), which are more common

among developing countries. Furthermore, some genetic factors also increase the CKD risk, such as Alport's syndrome, polycystic kidney disease and the presence of two APOL1 risk allele, that are mostly common amongst African Americans [1], [9], [15], [16].

GFR is the amount of plasma filtered by the Bowman's capsule per unit of time [5], and it is used to evaluate kidney function. This parameter may be determined by exogenous filtration markers, as insulin, or endogenous filtration markers, as creatinine and cystatin C. Although the usage of exogenous filtration markers allows a good GFR measure, insulin is not commonly used because of its elevated cost. A cheaper alternative is to use the endogenous filtration markers to estimate GFR, however they do not guaranty the same precision and accuracy of the results. On one hand, creatinine values are affected by other factors including changes in muscle mass or drug intake. On the other hand, cystatin C values are influenced by age and sex, as well as by the presence of inflammation or corticosteroids intake, which may increase its concentration. Therefore, to improve the accuracy of estimated glomerular filtration rate (eGFR) values, clinical practice may combine cystatin C and creatinine values by using a well-described formula by the new KDIGO guidelines (2012), known as the CKD Epidemiology Collaboration (CKD-EPI) equation (Equation 2.1) that considers both cystatin C and creatinine concentration, age, sex and ethnicity [6], [15]:

$$\begin{aligned} eGFR = 135 \times \min(SCr/\kappa,1))^{\alpha} \times \max(SCr/\kappa,1)^{-0.601} \times \min(SCysC/0.8,\ 1)^{-0.375} \\ \times \max(SCysC/0.8,1)^{-0.711} \times 0.995^{Age}\ [\times 0.969 \text{ if female}]\ [\times 1.08 \text{ if black}], \end{aligned} \quad \textbf{(2.1)}$$

where SCr stands for serum creatinine (mg/dl), SCysC is serum cystatin C (mg/l), κ is equal to 0.7 for females and 0.9 for males, α is -0.248 for females and -0.207 for males, min(SCr/κ,1) is the minimum of SCr/κ or 1, max(SCr/κ,1) is the maximum of SCr/κ or 1, min(SCysC/κ,1) is the minimum of SCysC/κ or 1 and max(SCysC/κ,1) is the maximum of SCysC/κ or 1 [6].

Another diagnosis method to assess kidney damage consists of proteinuria measurement, meaning an abnormal amount of proteins present in the urine. This parameter can be determined by dipstick qualitative methods to calculate the protein to creatinine ratio or the albumin to creatinine ratio (ACR) [1]. Hence, in daily clinical practice, in case the eGFR is normal, the ACR test is preformed, preferably with morning urine samples, to help assess kidney damage and possibly detect CKD [1], [15]. In this regard, in terms of kidney function, a CKD patient is characterized by an eGFR lower than 60 mL/min/1.73 m^2 or by an ACR higher than 30 mg/g in urine [9].

Despite these diagnosis methods being widely used in clinical practice, they do not inform on the etiology of the disease or the grade of tissue damage. Hence, percutaneous kidney biopsy is a necessary method to confirm the diagnosis and to identify the activeness and chronicity of the disease [15]. Although this method is considered the gold standard method for renal diseases diagnosis, it is an invasive procedure that entails potential risks and complications, such as severe bleeding, hematuria, urethral obstruction or even death. However, for patients with high risk of bleeding, transjugular and laparoscopic approaches may be alternatives to kidney biopsy [15]. In addition, to confirm the etiology of the renal disease, imaging techniques, such as radiography, echography or computer tomography may be used to differentiate inherent causes of kidney disease and to identify hereditary or congenital renal diseases [1], [15].

After the diagnose, the disease is categorized according to KDIGO classification, based on GFR or on the albuminuria state, as shown in Table 2.1. Concerning the GFR, the disease can

be divided into six stages. In stage 1, the GFR and kidney function are well preserved, but in stage 2 the GFR has already been reduced to values between 60 and 89 mL/min/1.73 m^2. Stage 3 can be subdivided into two substages, both with visible kidney damage, stage 3a where the GFR decrease to levels between 45 and 59 mL/min/1.73 m^2 and stage 3b, where the GFR is comprised between 30 and 44 mL/min/1.73 m^2. In later stages, there is a significant kidney damage, varying from 15 to 29 mL/min/1.73 m^2 in stage 4 and being under 15 mL/min/1.73 m^2 in stage 5, implying that the patient suffers from kidney failure [6], [9], [15]. Relatively to the albuminuria, CKD can be divided into three stages. In stage A1, the ACR is lower than 30 mg/g. In stage A2, the ACR is between 30 mg/g and 300 mg/g, which indicates that kidney function has already decreased, and albumin is starting to be accumulated in the urine. Finally, in stage A3, the ACR is higher than 300 mg/g, resulting in kidney failure [6], [9].

Table 2.1 - Classification of CKD based on GFR and albuminuria according to KDIGO.

GFR category	GFR (mL/min/1.73m^2)	ACR category	ACR (mg/g)
G1	≥ 90	**A1**	< 30
G2	60 – 89		
G3a	45 – 59	**A2**	30 – 300
G3b	30 – 44		
G4	15 – 29	**A3**	> 300
G5	< 15		

2.1.1 – Chronic Kidney Disease Treatment

Kidney function declines along with disease progression, resulting in an accumulation of metabolic waste products in the blood as creatinine and urea, leading to the appearance of other comorbidities or ultimately, to death. Therefore, patients with poor kidney function must undergo in a RRT in order to remove accumulated toxins from circulation, such as kidney transplantation or dialysis treatment [6].

According to SPN, Portugal has the highest RRT prevalence in Europe and by the end of 2020, approximately 2011 per million population (pmp) were under some type of RRT [17].

Kidney transplant would be the most beneficial option for patients with kidney failure, but the wait list may last from months to years and not all patients are suitable for this option [1], [15]. Some of the requirements for a patient to be eligible for a renal transplantation are [1], [18]:

1. Must be classified as CKD stage 4-5 and/or be on RRT.
2. Must be physical and emotional ready to get through the transplant.
3. Must be financial ready and have an appropriate insurance to help cover all the expenses of the transplant and after the transplant.
4. Must be well educated about the cares the post-transplant requires.
5. Must not have any comorbid condition that may affect the transplant.

Living donors must also meet certain requirements, such as be between 18 and 70 years old, do not have any active malignancy or infection, have a body mass index under 35 kg/m, be HIV negative, do not have diabetes, albuminuria or hypertension and have a great kidney function (GFR higher than 80 mL/min/1.73 m^2) [19].

Another consideration that must be taken in account is the compatibility of the organ, as the rate of organ rejection/failure is 4% and 3% from deceased and living donors within a year, respectively [20]. Furthermore, despite the transplanted kidney being fully functional, it has a half-life of approximately 20 years, meaning that these patients need to be submitted to dialysis treatment during their lifespan [1].

Another modality of RRT is dialysis that consists of removing the excess of toxins and water from the blood by filtration through a semipermeable membrane. Depending on the filtration method, it is possible to classify dialysis treatment into hemodialysis or peritoneal dialysis (PD) [1], [21].

Hemodialysis has been used for decades and has proven to be an efficient method to replace renal function. Thus, according to SPN, 12458 patients were being treated by hemodialysis in Portugal in 2020, of which 20.1% were actively waiting for a renal transplant [17]. To perform this technique, the patient is connected to a dialyzer, often referred to as an "artificial kidney", by two catheters located in the arm, [1], [21], [22]. The procedure consists of pumping the blood through an arterial catheter into the hemodialyzer, where it is filtered, purified and pumped back into the body under positive pressure through the venous catheter, as shown in Figure 2.1. The artificial kidney is composed by capillary tubes where the blood and the dialysis fluid are separated by a semipermeable membrane circulating in opposite directions, allowing an efficient diffusion of the waste products and the excess of water from the blood into the dialysis fluid [1], [21], [22].

The most common problems associated to this therapy are related with the vascular access whether because of the infections or the obstructions. Besides, rapid ultrafiltration of the fluid can cause the vascular depletion to be greater than the reposition, promoting cardiovascular instability. In addition, thrombin adsorption to the catheter is the primary reason of thrombosis among patients undergoing hemodialysis treatment [22]. All of this leads to a mortality rate of about 14% in hemodialysis patients, mainly caused by cardiovascular events [17].

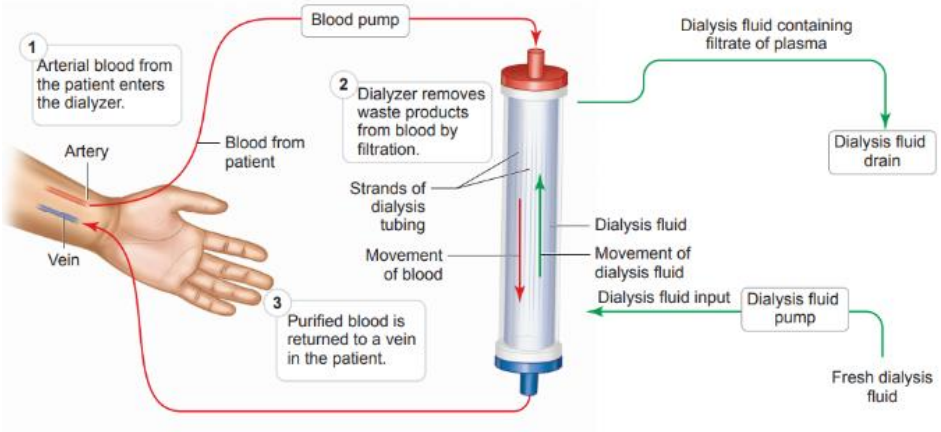


Figure 2.1 - Scheme of hemodialysis procedure: the arterial blood is transported into the hemodialyzer to be purified and returns to the veins under positive pressure. Adapted from [23].

The PD treatment is a less common method than hemodialysis and by the end of 2020, 878 patients were under PD treatment in Portugal, among which 50.6% were actively waiting for a renal transplant [17]. The technique consists of introducing through a catheter a PD fluid into the peritoneal cavity of the patient, where the peritoneum acts as a semipermeable membrane that allows the elimination of waste products and the excess of water from the blood. After a

residence time, that allows the transportation of the toxins from the blood to the dialysis fluid, it is drained out through the catheter, eliminating the waste products [24], as shown in Figure 2.2. One of the main disadvantages of PD is that it carries a greater risk of infection and of developing a hernia due to the insertion of the catheter [25]. Furthermore, the biocompatible nature of the dialysis fluids could provoke alterations on the peritoneum leading to an ultrafiltration failure, making the technique unfeasible. Besides, PD is a risk factor for emergency hospitalization and mortality, associated with cardiovascular diseases, that are the main cause of death of these patients, which present a mortality rate of about 5.4% [17]. On the other hand, this technique has some advantages as it allows a better preservation of the residual renal function, is cheaper than hemodialysis and offers more freedom to the patient, as it can be performed at home [25], [26].

Within the PD, there are also various modalities. The automated PD (APD) can be done with a cycler that performs automated cycles of multiple short fluid interchanges during night, and it is more indicated for patients with high transport status. Another type of PD is the continuous ambulatory PD (CAPD) that is done manually, without the need of a machine, and can be performed anywhere if the environment is clean [27], [28]. Comparing both types, there are no significant differences in survival and mortality rates, as well as peritonitis complications [27], [29]. In 2020, of the 878 patients that were under PD treatment in Portugal, 365 were on APD and 513 were on CAPD [17].

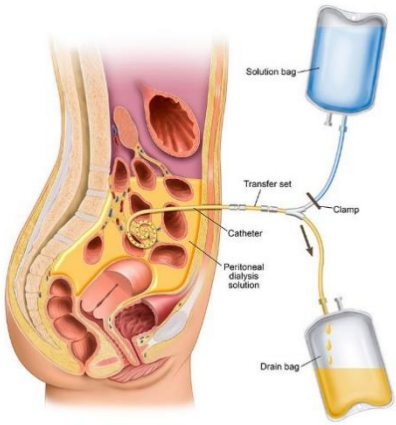


Figure 2.2 - Scheme of PD procedure: the PD fluid enters the peritoneal cavity where it attracts waste products and the excess of water using the peritoneum as a filter. After a time of residence, PD fluid is drained. Adapted from [30].

2.1.2 - Inflammation and Fibrosis in Chronic Kidney Disease

Inflammation and fibrosis are the driving force behind all the changes produced during renal damage, regardless of their origin [31].

Inflammation is a characteristic defense response to an injury that promotes tissue regeneration by stimulating the innate and adaptive immune system response, but the prolonged exposure to inflammatory triggers drives to a hyperactive and chronic inflammatory state that culminates with maladaptive tissue repair that triggers a pathological tissue fibrotic response [3], [32]. As mentioned earlier, diabetes and hypertension are the main contributors for CKD progression, as both diseases injure the renal blood vessels leading to kidney tissue destruction [33]. Hereby, damaged kidney cells release pro-inflammatory cytokines that

promote inflammatory cells infiltration, such as lymphocytes, dendritic cells and monocytes, which differentiate into M1 and M2 macrophages into the injured site [3], [34].

Therefore, these inflammatory cells produce profibrotic cytokines and growth factors, such as transforming growth factor beta 1 (TGF-β1), which leads to fibroblasts activation into myofibroblasts and to tubular epithelial cells (TECs) suffering a process called epithelial to mesenchymal transition (EMT) as shown in Figure 2.3 [32], [34]. However, it is important to note that under healthy conditions, after repair and regeneration of the injury, myofibroblasts suffer apoptosis, but under fibrotic conditions the myofibroblasts activation becomes uncontrolled, producing an excessive amount of extracellular matrix (ECM) components, contributing to a fibrogenic environment [3], [34]. Thus, fibrosis is the final pathological response to a maladaptive tissue repair that results from an excessive production and deposition of ECM components [3].

There are several profibrotic cytokines and growth factors interconnected to each other that trigger signaling cascades that lead to fibrosis. The renin-angiotensin-aldosterone system (RAAS) is a widely known system in the kidney for being responsible for the regulation of extracellular volume and arterial pressure. Briefly, renin is secreted by the juxtaglomerular cells of the kidney to produce angiotensin-I that is converted by the angiotensin-converting enzyme (ACE) into angiotensin-II (Ang-II) [3], which upregulates the expression of transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), tumor necrosis factor alfa (TNF-α) and nuclear factor kappa B (NF-κB), promoting renal fibrosis. TNF-α increases inflammation by stimulating the release of monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β) and TGF-β, wherein its isoform TGF-β1 promotes ECM components production and reduces the production of ECM-degrading proteins, resulting in an excessive accumulation of ECM [32], [33]. TNF-α also enhances the proliferation of fibroblasts and myofibroblasts and activates the TGF-β/Smad3 signaling cascade that leads to CTGF overexpression, which can regulate fibrosis through small mothers against decapentaplegic (Smad), extracellular signal-regulated kinases (ERK) and wingless integration site (Wnt) signaling pathways [32]. Another important contributor is C-reactive protein (CRP), an acute-phase protein capable of provoking the infiltration of inflammatory cells, resulting in the release of pro-inflammatory cytokines and growth factors, such as MCP-1, by activating TGF-β1, ERK/p38 and NF-κB signaling pathways [35]. Increased levels of these circulating cytokines have been identified under fibrotic conditions and predict poor renal outcomes, but the translation into the clinical set has not yet been achieved [32], [33].

The better understating of the cellular and molecular pathways involved in the crosstalk between renal fibrosis and the inflammatory microenvironment is of major relevance for the discovery of novel early biomarkers and therapeutic targets for CKD.

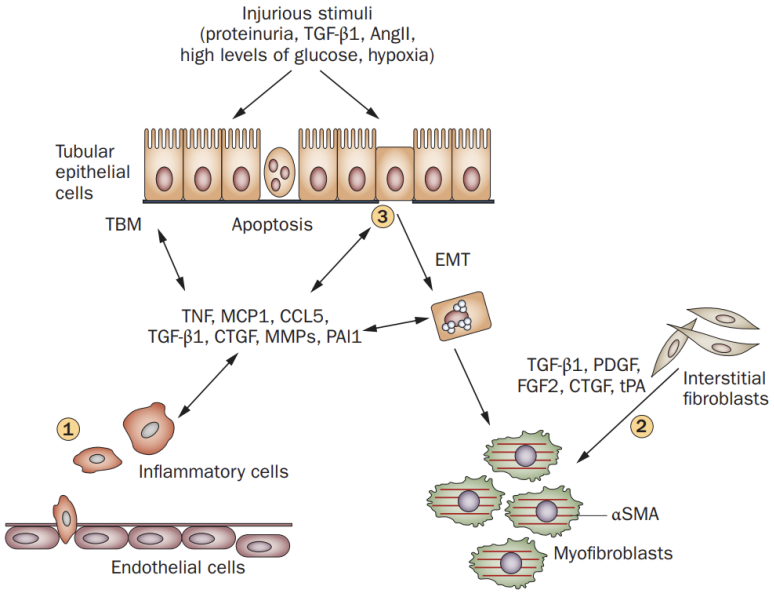


Figure 2.3 - Scheme of fibrosis: **(1)** Injured endothelial cells promote inflammatory cells infiltration that release profibrotic growth factors, such as TGF-Β1, **(2)** which promote the activation of fibroblasts and TECs into myofibroblasts and **(3)** EMT processes. Myofibroblasts produce an excessive amount of ECM proteins, that results in a maladaptive tissue repair, fibrosis. Adapted from [34].

2.2 - Cardiovascular Outcomes in Chronic Kidney Disease

Kidneys regulate several biological processes by keeping a balanced concentration of the several components of the organism. However, upon renal failure, there is a dysregulation that affects many organ systems, causing several systemic complications including an increase in cardiovascular risk [15].

There is an established bidirectional relationship between the kidneys and the heart that explains the interconnection between CKD and CVD that can worsen each other's progression. This relationship was first described by Dr. Richard Bright in 1836 and justified by the presence of common risk factors, such as hypertension and diabetes [2], [36], [37].

Besides the two common risk factors, these two organs are intertwined by neurohormonal pathways, such as the sympathetic nervous system (SNS) and the RAAS [38], [39]. Thus, when heart failure occurs, there is a reduction in the cardiac output that causes a decrease in renal perfusion, which aggravates renal function [39], [40]. This decrease in renal perfusion is detected by juxtaglomerular cells that activate the RAAS cascade in order to maintain a normal perfusion and GFR [38], [39], [41]. This cascade begins with renin release in response to low blood pressure, which leads to the production of Ang-II that triggers the activation of SNS and causes the constriction of the afferent and efferent arterioles of the glomerulus and promotes aldosterone release. Aldosterone contributes to sodium and water reabsorption and promotes vasoconstriction of blood vessels, increasing renal vascular resistance and blood pressure, creating a volume overload that reaches the heart. This increase causes an augmentation in cardiac frequency in response to elevated pressure to increase renal blood flow, which together with overstimulation of the neurohormonal pathway promotes the progression of kidney disease and heart failure [38], [39].

Therefore, CKD represents a major independent risk factor for CVD, increasing the risk of developing a cardiovascular condition up to 500-fold [2]. It is noteworthy that the cardiovascular risk is increased by 43% in CKD patients in stage 3 and by 343% in stage 5 [2], [6]. In addition, GFR decline, and albuminuria are also promoters of several cardiovascular outcomes, such as heart failure, myocardial infarction (MI), stroke, coronary heart disease, sudden cardiac death and left ventricular hypertrophy, among others [37]. A GFR declining below 45 mL/min/1.73 m^2 with normal albuminuria or the presence of albuminuria, even in the A1 range, with normal GFR, entails a significant increase in cardiovascular risk. Furthermore, if both conditions occur, at the same time, they markedly potentiate the development of cardiovascular events [36], [42] Thus, CKD, GFR and albuminuria are strong independent risk factors of CVD [36], [37], [42]. To understand the impact that CVD has on CKD, it is noteworthy that heart failure is the main cause of death among these patients, being responsible for 50% of all deaths [2], [40]. Besides, if we compare patients with normal renal failure that suffer from CVD with individuals that suffer from both comorbidities, the latter presents higher mortality rates of about 58-71% [37].

Though traditional risk factors are the main cause of both diseases, nontraditional risk factors are responsible for higher death rates among CKD individuals with CVD, including dyslipidemia, vascular calcification, endothelial dysfunction, inflammation, and oxidative stress [36], [37].

Dyslipidemia is characterized by an increased amount of low-density lipoproteins cholesterol (LDL-C), triglycerides and apolipoprotein B and by low levels of high-density lipoprotein cholesterol (HDL-C) and total cholesterol. This dysregulation in lipid metabolism is common among CKD patients, and it is one of the consequences of GFR declining. An advanced stage of dyslipidemia is accompanied by elevated levels of LDL-C that deposits within the tunica intima of the arteries, causing atherosclerosis and endothelial dysfunction, increasing cardiovascular mortality [43]–[45]. In the case of vascular calcification, there is a loss of elasticity of the arteries, increasing the stiffness of the tunica intima and media of the arteries' walls. CKD actively contributes to the progression of vascular calcification under the influence of several risk factors, such as mineral bone disorder, dyslipidemia, age and sex. Mineral bone disorder arises as a consequence of an imbalance in the calcium and phosphate levels in CKD patients. In early stages of CKD, fibroblast growth factor 23 (FGF23) levels are increased, which inhibits the calcitriol absorption into the organism, resulting in a decrease of calcium and phosphorus adsorption and in an increased synthesis of parathyroid hormone, provoking their release from bones. Furthermore, continuous increased levels of FGF23 inhibits phosphate adsorption causing hyperphosphatemia, which induces the calcification of vascular smooth muscle cells, contributing to vascular calcification [46], [47]. Endothelial dysfunction is characterized by an imbalance between vasoconstriction and vasodilation and affects the permeability of the endothelial layer. This condition is accompanied by elevated expression of pro-inflammatory factors and decreased levels of nitric oxide bioavailability and its pathophysiology involves chronic inflammation and oxidative stress [48], [49]. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and their degradation rate. It has been shown that CKD patients present elevated ROS concentrations due to impaired renal function that in association with LDL-C, can provoke its oxidation originating oxidized LDL-C, which promotes atherosclerosis. In addition, an increase in ROS levels causes endothelial dysfunction and reduces the nitric oxide availability. These ROS are also a major contributor of the inflammatory response as oxidative stress can induce the activation of NF-κB pathway

and the synthesis of CRP, TNF-α and interleukin-6 (IL-6) [50], [51]. All of these nontraditional risk factors are common for CKD and CVD, and are also interconnected between them, affecting one another.

All these evidences reveal that the heart and the kidney are intrinsically correlated, sharing many risk factors and influencing one and another, but the pathophysiological mechanisms underlying this connection remain largely unknown and further investigations are necessary.

2.2.1 - Cardiac Fibrosis

When the heart suffers an injury, such as MI, cardiac fibroblasts (CFs) respond by transforming themselves into myofibroblasts and damaged blood vessels help the tissue to recruit inflammatory cells including mast cells, macrophages and lymphocytes. These cells promote the secretion of pro-inflammatory cytokines to activate tissue repair mechanisms, but the prolonged exposure to such environment leads to a maladaptive response culminating with a pathological fibrotic response [52]–[54].

The abovementioned nontraditional risk factors strongly affect the heart and can lead to cardiac fibrosis. For instance, increased levels of dyslipidemia, that is defined by an accumulation of lipidic molecules in the walls of large arteries, difficults the blood to flow through them, and leads to pressure overload and further vascular damage [45], [55]. In addition, CKD patients suffer from mineral bone disorder, which is an abnormality of mineral metabolism related to vascular calcification, which results in a dysregulated synthesis of FGF23, leading to cardiac afterload and hypertrophy. Taken together, these two dysfunctions promote heart failure and contribute to the setting of cardiac fibrosis [46], [56]. However, there are several other factors that contribute to vascular damage and heart failure that can induce cardiac fibrosis.

In the setting of cardiac fibrosis, RAAS is continuously activated creating an overexpression of its downstream effectors, namely Ang-II and aldosterone. Ang-II is responsible for high blood pressure and vasoconstriction, which worsens the heart condition damaging the tissues. In addition, Ang-II induces the expression of TGF-β1 by fibroblasts and cardiomyocytes, which regulate many inflammatory and fibrotic signaling pathways. The final effector of this neurohormonal system, aldosterone, is also implicated in several fibrotic pathways by inducing the release of more pro-inflammatory cytokines, such as TNF-α via NF-κB signaling pathway, and by increasing the proliferation of CFs and the subsequent collagen synthesis. Furthermore, aldosterone stimulates cardiomyocytes to activate the regulation of matrix metalloproteinase-2 (MMP-2), which further activates TGF-β1 signaling pathway. The constant upregulation of these fibrotic factors contributes to an excessive production of ECM components, vascular calcification and impaired diastolic filling of the heart, which further contribute to the development of cardiac fibrosis [52]–[54].

It is stated that CKD patients have more fibrotic tissue in the heart in comparison with patients without CKD. This pathology is characterized by an increase of ROS that play a key role inducing the fibrotic response. TNF-α promotes the accumulation of these molecules and also the activation of TGF-β pathway that contributes to collagen type I and type II deposition and, in consequence, to fibrosis. In addition, this cytokine regulates mitogen activated protein kinase (MAPK) signaling cascade and the RAAS, establishing a relationship between inflammation, oxidative stress and RAAS, and facilitating the interconnection between cardiac fibrosis and CKD. Moreover, the pro-inflammatory cytokine IL-1β is upregulated in CKD that

results in an increase of TGF-β1 signaling pathway and in an increased expression of collagen type I [57].

Kidney and heart fibrosis share many inflammatory and fibrotic pathways, thus the existence of common pathophysiological mechanisms involved in kidney-heart injury are suspected [57]. However, the mechanisms underlying cardiorenal dysfunction that occurs during CKD are unknown and new approaches are needed to ameliorate the diagnosis and treatment of these diseases. In this sense, the possibility of a kidney-heart cross talking messengers offers a wide field to discover, and miRNAs could be one of these linkers.

2.3 - MicroRNAs

MiRNAs are small endogenous, non-coding RNAs (19-22 nucleotides) originated from the double-stranded region of an RNA hairpin precursor of 60-70 nucleotides and their coding genes are localized in exons of noncoding genes, introns of coding or noncoding genes and in intergenic regions [4], [58], [59]. These molecules were first discovered in 1993 in *Caenorhabditis elegans* by Lee and colleagues, but only in 2001 they rose interest amongst the scientific community, when miRNAs were found in *C. elegans*, *drosophila* and human cells [59]. Its primarily function is to regulate gene expression at a post-transcriptional level, without modifying the genetic code, as they target the 3´untranslated region (UTR), the 5´UTR and act as gene promoters by degrading messenger RNA (mRNA) or inhibitors of its translation, resulting in a decrease of expression levels [4], [60]. It is estimated that 30% of protein-coding genes are modulated by miRNAs, and currently, 38589 miRNAs have been identified and organized in a public database (miRBase) [61] with their respective hairpin and mature sequences [58].

These small nucleic acids are present intracellularly, but recent reports have found that a large number of miRNAs are present in extracellular body fluids, including plasma, urine, peritoneal fluid and saliva, among others [4], [60]. The miRNAs in these fluids are known as circulating miRNAs and differ from traditional RNA molecules and intracellular miRNAs, as they are highly stable and resistant to RNase activity and to large variations of temperature and pH, as a result of their encapsulation in lipid vesicles – exosomes – or in building complexes with RNA-binding proteins. This encapsulation protects miRNAs from circulating ribonucleases present in body fluids, while carrying them from one cell to another allowing a cell-to-cell communication [4], [60], [62].

Both intracellular and circulating miRNAs hold important structural, regulatory and catalytic functions and consequently, participate regulating several biological processes. For instance, they have been described to play key roles in cell development, differentiation, cycle regulation and apoptosis processes. In this sense, as miRNAs participate in such important processes, its aberrant expression is usually related with pathological conditions [62].

2.3.1 – MicroRNA Biogenesis

The miRNAs' biogenesis starts within the nucleus, as shown in Figure 2.4, and is categorized into canonical and non-canonical pathways, whereby the first one is the predominant pathway [63].

In the canonical pathway, the miRNA gene is transcribed by RNA polymerase II into a long stem-loop primary miRNA (pri-miRNA). Then, this pri-miRNA is transformed into a precursor miRNA (pre-miRNA) by a multiprotein complex called Microprocessor, composed of Drosha and

DiGeorge syndrome critical region gene 8 (DGCR8), to be further exported to the cytoplasm by Exportin-5. In the cytoplasm, the terminal loop of the pre-miRNA is cleaved by Dicer, a RNase III enzyme, that together with other double-stranded RNA-binding proteins, a protein kinase RNA activator and a transactivation response RNA-binding protein (TRBP), finally produce the mature miRNA duplex. Afterwards, the two miRNA strands are separated based on thermodynamic asymmetry resulting in a guide or 5p strand that derives from the 5' end, and a passenger or 3p strand that arises from the 3' end. The guide strand is very unstable and is incorporated into the RNA-induced silencing complex (RISC), which is produced by RNA-binding proteins associated with Argonaute (Ago) proteins, whereas the passenger strand is cleaved by Ago2 proteins and further degraded. Hence, the guide strand leads the complex to its targets where assembles the mRNA at the 3' UTR by sequence complementarity, resulting in the inhibition of the translation or mRNA degradation [4], [63], [64].

There are several non-canonical pathways for miRNA biogenesis, such as miRtron pathway, small nucleolar RNA-derived pathway, transfer RNA pathway and Dicer-independent pathway, but the most common is the miRtron pathway [65]. In this pathway the miRNA gene is transcribed into a short hairpin intron known as miRtron by RNA polymerase II and further cleaved by spliceosomes, originating a branched pre-miRNA. Afterwards, the pre-miRNA is debranched by the debranching enzyme 1 and refolded into a stem-loop structure to be exported by Exportin-5 to the cytoplasm. Once in the cytoplasm, the miRNA's biogenesis continues in a similar way to the canonical pathway [65]–[67].

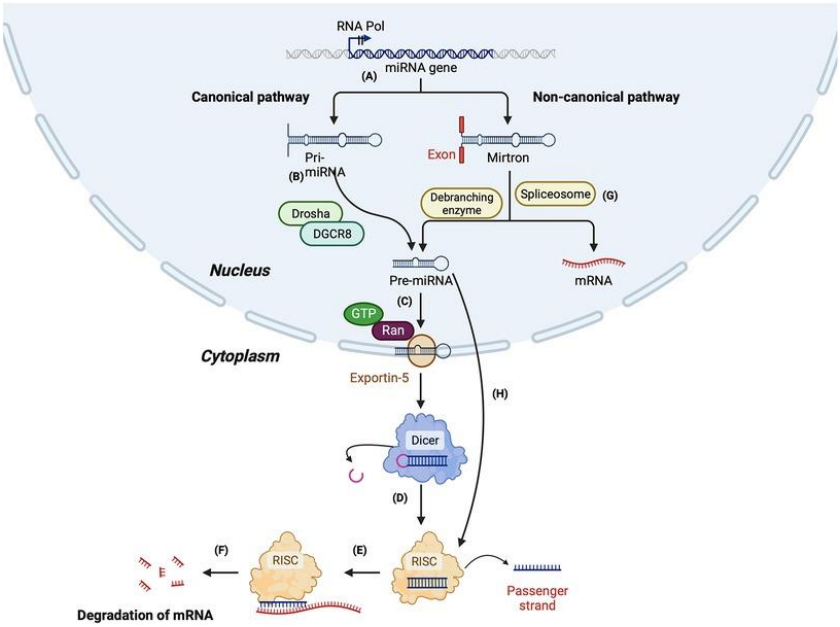


Figure 2.4 - Scheme of canonical and non-canonical pathways of miRNAs' biogenesis. Canonical pathway: **(A)** miRNA gene is transcribed into pri-miRNA and **(B)** it is processed by Drosha/DGCR8 complex into a pre-miRNA to be further **(C)** exported to the cytoplasm by Exportin-5. In the cytoplasm, **(D)** the Dicer enzyme cleaves the stem-loop to produce a mature miRNA duplex. Then, **(E)** the passenger strand is degraded, while the guide strand is incorporated into the RISC complex to **(F)** guide it to target mRNA to degrade it or inhibit its translation. Non-canonical pathway: **(G)** The pri-miRNA is processed by spliceosomes into a branched pre-miRNA, which is further debranched by debranching enzyme 1 and then **(H)** the biogenesis process is similar to the canonical. Adapted from [68].

2.3.2 - MicroRNAs as Diagnostic Biomarkers

A diagnostic biomarker is defined as an indicator that detects or confirms a certain pathological condition. Hence, if the expression pattern of a miRNA becomes dysregulated during a pathological process, this fact turns these molecules into suitable candidates for being diagnostic biomarkers. In 2008, miRNAs were first described as diagnostic biomarkers for cancer detection, when a group of scientists used them to study the diffusion of large B-cell lymphoma, giving rise to numerous investigations in other diseases among the scientific community [60], [68].

Another feature that makes circulating miRNAs good biomarker candidates is that they are highly stable in extracellular fluids and easily detected by basic molecular biology techniques, allowing to measure alterations that could be associated with the beginning or progression of human disorders and diseases [60], [69]. In addition to these parameters, there are some other characteristics that must be fulfilled by miRNAs to be good biomarkers, such as [68], [70]:

1. Allow a non-invasive, quick, and inexpensive diagnose.
2. Be present in accessible sources to be easily measured.
3. Give information about possible outcomes and the underlying mechanisms of the disease.
4. Have a high sensitivity to allow a precise and early detection.
5. Have a high specificity so as not to be affected by other pathologies.
6. Respond to treatment and to the progression of the disease.

Taking these requirements into consideration, on certain occasions, more than one biomarker can be used to complement each other and provide a better insight of the disease and a better diagnosis.

Thus, a good biomarker needs to be non-invasive, which means that it should be easily accessible without causing severe complications for the patient. As mentioned before, CKD is diagnosed by performing a kidney biopsy, an invasive procedure with several risks for the patient so, to reduce such complications, miRNAs would be good biomarker candidates as they can be retrieved from readily available sources, such as blood and urine, which are easily collected from the patient and do not require invasive procedures [68], [70].

Moreover, the biomarker's detection method should be low cost and quick to detect to allow the assessment of several samples and provide rapid results without imposing a huge expenditure for the testing facilities. Hence, miRNAs can be detected by northern blotting, which is a low throughput and time-consuming technique, by qRT-PCR, a highly sensitive and specific method or by microarray that offers a quick and high throughput detection, but is an expensive technology. However, new detection technologies based on nanomaterials are being developed to provide more efficient and cost-effective detections [68], [71].

In addition, a good biomarker should provide information about possible outcomes of the disease, which means that it should help predict disease progression, recurrence and prognostic. As miRNAs regulate several developmental processes including differentiation, apoptosis and proliferation, among others, a single miRNA may be implicated in several common physiological mechanisms. Thereby, the comparison of the expression pattern between patients at different stages of the disease may help with the correlation of disease progression and the combinatorial analysis of several miRNA profiles may provide a better evaluation of the disease outcome. Besides, it would be desirable for a biomarker to provide

information about the underlying mechanisms of the disease by giving more insightful details about the pathways and molecules involved in the disease [62], [68].

Another important requirement is that the biomarker should be cell or tissue-specific and sensitive to disease progression. Receiver operating characteristic (ROC) curves are very useful to measure these features as they plot the sensitivity in function of the specificity at several cut-off points. This analysis uses the area under the ROC curve (AUC) to compare the performance between two or more diagnostic groups in order to select the ideal for clinical use, where an AUC of 1.0 represents a perfect biomarker performance [70], [72], [73]. Even though several miRNAs are involved in many metabolic pathways, certain miRNAs are tissue- and disease-specific. The sensitivity of a miRNA allows the early detection of the disease, given that a slightly change in the normal condition is exhibited as a significant alteration of the expression pattern and the specificity of a miRNA allows the expression pattern not to be affected by the presence of other comorbidities, providing an accurate detection of the condition [74], [75].

Finally, a good biomarker should vary according to treatment and disease progression to provide more insightful information about the stage of the disease. MiRNA expression patterns can be altered with disease progression, allowing the identification of the stage of the disease. In addition, miRNAs respond to treatment and therefore, the identification of these levels may help assess treatment efficacy and help guide treatment-related decisions to come up with patient-tailored therapies and provide better outcome results [68], [70].

2.3.3 - MicroRNAs Involved in Kidney and Cardiac Fibrosis

MiRNAs regulate several biological processes and fibrotic processes are not an exception. They regulate many fibrotic signaling pathways that are not only implicated in renal fibrosis or cardiac fibrosis, but may affect several organs. In addition, their expression pattern is dysregulated under pathological conditions therefore, it would be of major importance to investigate miRNA expression and signaling pathways to better understand the fibrotic pathophysiology that may occur in both heart and kidney during CKD. In this sense, several miRNAs have already been described to be involved in CKD and CVD alone and it is obvious that some miRNAs intervene in both pathologies at it is shown in Table 2.2.

Table 2.2 - MicroRNAs involved in kidney and heart fibrosis.

MicroRNA	Pathology	Model	Expression	Pathway	Ref.
miR-21	CKD	Streptozotocin (STZ)-induced diabetic ApoE$^{-/-}$ mouse, STZ-induced uni-nephrectomized Sprague Dawley (SD) rats and C57B1/6 mice with adenine-induced renal fibrosis (*in vivo*), kidney tissue from biopsies of patients diagnosed with diabetic nephropathy (DN) and rat proximal TECs treated with TGF-β1 (*in vitro*)	Up	TGF-β1/Smad3	[76]

miR-21-5p	CVD	Normal, Smad2 or Smad3 knockdown (KD) rat TECs line (NRK52E) (*in vitro*), Smad2 or Smad3 knockout (KO) mouse embryonic fibroblasts (MEFs) treated with human TGF-β1 (*in vitro*), unilateral ureteral obstruction (UUO) induced in Smad3 wild-type (WT) and KO mice or conditional Smad2 KO mice (*in vivo*)	Up	TGF-β1/Smad3	[77]
		Mice with UUO kidneys (*in vivo*) and human kidney 2 (HK-2) cells treated with TGF-β1 or TNF-α or aristolochic acid (*in vitro*)	Up	TGF-β1	[78]
		C57BL/6 mice with induce MI (*in vivo*) and CFs from SD rats treated with TGF-β1 (*in vitro*)	Up	TGF-β1/Smad3	[79]
		H9C2 cells subjected to hypoxia transfected with miR-21 mimics or miR-21 inhibitor (*in vitro*)	Up	TGF-β1/Smad3	[80]
		SD rats with induced MI (*in vivo*) and CFs treated with TGF-β1 or treated with adenovirus that carried miR-21 inhibitor (*in vitro*)	Up	TGF-β1	[81]
	CKD	UUO C57BL/6 mice (*in vivo*), human TEC line HK-2 (*in vitro*)	Up	SPRY1/ERK/NF-κB	[82]
		Human kidney biopsies from patients diagnosed with IgA nephropathy (*in vivo*)	Up	TGF-β1	[83]
	CVD	Hypertrophic cardiomyopathy patients with myocardial fibrosis (*in silico*)	Up	TGF-β1/Smad3	[84]
miR-26a	CKD	UUO C57BL/6J mice with induced renal fibrosis after intramuscular injection of exosome-carried miR-26a (*in vivo*)	Down	TGF-β1	[85]
	CVD	CFs pretreated with SN50 and IkBα followed by stimulation with Ang-II (*in vitro*)	Down	NF-κB	[86]
		SD mice with induced MI (*in vivo*) and human CFs transfected with miR-26a	Up	PI3K/AKT	[87]

		mimics or miR-26a inhibitor (*in vitro*)			
miR-29	CKD	UUO nephropathy induced in Smad3 WT and KO mice (*in vivo*), Smad3 WT MEFs (*in vitro*), Smad3 KD TEC line NRK52E (*in vitro*)	Down	TGF-ß1/Smad3	[88]
	CVD	C57BL/6 N mice deficient of miR-29 (miR-29 ab1$^{-/-}$b2c$^{+/-}$) (*in vivo*) and cardiac myocytes transfected with antimir-29 (*in vitro*)	Up	Wnt	[89]
miR-29a	CKD	WT friend virus B mice with induced diabetes by STZ (*in vivo*), mouse CV40 MES-13 glomerular mesangial cells treated with high glucose (HG) or miR-29a inhibitor (*in vitro*)	Down	Wnt/ß-catenin	[90]
	CVD	SD rats with cardiac fibrosis induced by isoproterenol (*in vivo*), HEK293 cells and rat CFs transfected with miR-29c mimics (*in vitro*)	Down	Ras/ERK1/2	[91]
miR-29b		UUO CD-1 mice (*in vivo*), rat NRK-52E cells treated with Ang-II (*in vitro*)	Down	PI3K/AKT	[92]
	CKD	NRK-52E cells treated with Ang-II and transfected with miR-29b mimics or miR-29b inhibitor (*in vitro*)	Down	PI3K/AKT	[93]
	CVD	Smad3 WT mice with induced hypertensive heart disease by Ang-II (*in vivo*), CFs from Smad3 KO or WT mice treated with Ang-II and transfected with miR-29b mimics or miR-29b inhibitor (*in vitro*)	Down	TGF-ß1/Smad3	[94]
miR-29b-3p	CKD	BUMPT cells transfected with miR-29-3p mimics or miR-29b-3p inhibitor (*in vitro*)	Down	TGF-ß1	[95]
	CVD	CFs from SD rats treated with TGF-ß1 (*in vitro*)	Down	TGF-ß1	[96]
miR-29c	CKD	UUO mice (*in vivo*) and rat fibroblasts NRK-49F treated with TGB-ß1 and transfected	Down	Wnt/ß-catenin	[97]

		with miR-29c mimics or miR-29c inhibitor (*in vitro*)			
	CVD	C57Bl/6 mice with induced cardiac fibrosis by lipopolysaccharide (LPS) injection (*in vivo*) and CFs incubated with LPS (*in vitro*)	Down	TGF-ß	[98]
	CKD	C57BL/Ks db/db mice manipulated by rAAV-miR-30c and rAAV-anti-miR-30c (*in vivo*), mice TECs treated with rAAV-miR-30c or rAAV-anti-miR-30c and HK-2 treated with Ang-II and transfected with miR-30c mimics or miR-30c inhibitor (*in vitro*)	Down	Snail1/TGF-ß1	[99]
miR-30c	CVD	SD rats with induced atrial fibrosis by abdominal aortic constriction (*in vivo*) and CFs treated with TGF-ß1 and transfected with miR-30c mimics or miR-30c inhibitor *(in vitro*)	Down	TGF-ß1	[100]
	CKD	Human DN tissues (*in vitro*) and HK-2 cells treated with HG and transfected with miR-30c-5p mimics (*in vitro*)	Down	PI3K/AKT	[101]
miR-30c-5p	CVD	C57BL/6J mice with induced myocardial injury by administration of isoproterenol (*in vivo*)	Down	TGF-ß	[102]
	CKD	UUO mice (*in vivo*) and HK-2 transfected with miR-34a mimics (*in vitro*)	Up	TGF-ß1	[103]
miR-34a	CVD	C57BL6 mice with induced MI and injected with antagomir-34a (*in vivo*) and CFs treated with miR-34a mimics or miR-34a inhibitor (*in vitro*)	Up	TGF-ß1/Smad4	[104]
	CKD	UUO C57BL/6 mice treated with miR-101a agomir (*in vivo*) and HK-2 cells incubated with aristolochic acid (*in vitro*)	Down	YAP/ TGF-ß/Smad	[105]
miR-101a	CVD	SD rats subjected to transverse aortic constriction (TAC) injected with miR-101a mimics	Down	TGF-ß1	[106]

		(*in vivo*) and CFs stimulated by TGF-β1 and transfected with miR-101a mimics or miR-101a inhibitor (*in vitro*)			
		SD rats with induced MI by left anterior descending coronary artery (*in vivo*) and CF subjected to hypoxia (*in vitro*)	Down	TGF-β	[107]
	CKD	UUO C57BL/6 WT mice (*in vivo*) and NIH3T3 and C3H/10T1/2 cells treated with TGF-β and with antagomir-132 (*in vitro*)	Up	TGF-β	[108]
miR-132		SD rats with induced MI by coronary artery ligation treated with Ang-II and with miR-132 agomiR or miR-132 antagomiR (*in vivo*) and CFs treated with Ang-II and transfected with miR-132 agomiR or miR-132 antagomiR (*in vitro*)	Down	PTEN/PI3K/AKT	[109]
	CVD	Wistar rats with induced dilated cardiomyopathy (DCM) by intraperitoneal injection of doxorubicin (*in vivo*)	Down	PTEN/PI3K/AKT	[110]
miR-132-3p	CKD	C57BL/6J mice with acute kidney injury induced by cisplatin administration (*in vivo*) and HK-2 cells treated with cisplatin and transfected with miR-132-3p mimics or miR-132-3p inhibitors (*in vitro*)	Up	NF-κB	[111]
	CVD	Human CFs (*in vitro*)	Up	FOXO3a	[112]
	CKD	Mouse renal TECs treated with TGF-β1 and transfected with miR-146a-5p mimic (*in vitro*)	Down	TGF-β1	[113]
miR-146a-5p	CVD	Human AC16 cardiac cells stimulated with TNF-α transfected with miR-146a-5p mimics or miR-146a-5p inhibitor (*in vitro*)	Up	TGF-β/ Smad4	[114]
miR-199a-5p	CKD	Rat mesangial cells cultured with glucose and transfected with miR-199a-5p inhibitor (*in vitro*)	Up	TLR4/NF-κB p65/NGAL	[115]
	CVD	C57BL/6 mice with induced fibrosis by Ang-II infusion (*in*	Up	NF-κB	[116]

		vivo) and CFs transfected with miR-199a-5p mimics or miR-199a-5p inhibitor (*in vitro*)			
	CKD	OLETF rats with induced type 2 diabetes injected with LV-anti-miR-199b (*in vivo*), HK-2 treated with TGF-β transfected with miR-199b mimics or miR-199b inhibitor (*in vitro*)	Up	TGF-β	[117]
miR-199b					
	CVD	Mice subjected to TAC and mice subjected to pressure overload of the left ventricle (*in vivo*), rat neonatal cardiomyocytes transfected with miR-199b precursor (*in vitro*)	Up	Calcineurin/NFAT	[118]
	CKD	UUO C57BL/6 WT mice injected with LV-miR-214-KD (*in vivo*) and HK-2 cells treated with TGF-β1 transfected with miR-214-3p mimics (*in vitro*)	Up	PTEN/PI3K/AKT	[119]
miR-214-3p					
	CVD	C57BL/6J mice with induced MI by LAD and subjected to chronic intermittent hypoxia (*in vivo*)	Down	TGF-β	[120]
	CKD	Munich Wistar Frömter and Wistar rats treated with ACE inhibitor lisinopril (*in vivo*) and NRK cells treated with TGF-β and transfected with miR-324-3p mimics (*in vitro*)	Up	TGF-β	[121]
miR-324-3p					
	CVD	SD rats with induced atrial fibrillation by a calcium chloride-acetylcholine mixture injection (*in vivo*) and CF transfected with miR-324-3p mimics (*in vitro*)	Down	PI3K/AKT	[122]
	CKD	UUO Smad3 KO mice treated with miR-433 shRNA (*in vivo*) and NRK52E cells treated with TGF-β1 and transfected with miR-433 mimics or miR-433 inhibitor (*in vitro*)	Up	TGF-β/Smad3	[123]
miR-433					
	CVD	Heart tissues from patients with DCM, C57BL/6 mice with doxorubicin-induced	Up	TGF-β/Smad3	[124]

	cardiomyopathy administered with miR-433 antagomir (*in vivo*) and CFs from SD rats stimulated with TGF-β1 or Ang-II

Abbreviations: **AKT** – protein kinase B; **DCM** – dilated cardiomyopathy; **DN** – diabetic nephropathy; ERK - extracellular signal-regulated kinases; **HG** – high glucose; **HK** – human kidney; **KD** – Knockdown; **KO** – Knockout; **LAD** - left anterior descending coronary artery; **LPS** – lipopolysaccharide; **LV** – lentivirus; **MEFs** – mouse embryonic fibroblasts; **MI** – myocardial infarction; **NFAT** - nuclear factor of activated T-cells; **OLETS** - Otsuka-Long-Evans-Tokushima-Fatty; **PI3K** - Phosphoinositide 3-kinases; **rAAV** - Recombinant adeno-associated virus; **SD** – Sprague Dawley; **shRNA** – small hairpin RNA; **Smad** – small mothers against decapentaplegic; **STZ** – streptozotocin; **TAC** – transverse aortic constriction; **UUO** – unilateral ureteral obstruction; **Wnt** - Wingless integration site; **WT** – wild-type.

As shown in Table 2.2, TGF-β and its isoform TGF-β1 play a key role in renal and cardiac fibrosis. This cytokine is of extremely importance for fibrosis, but also for the development of biological processes. It induces fibroblast activation into myofibroblasts, which secrete several ECM compounds, induces the release of many fibrotic molecules and activates fibrotic signaling pathways. In addition, TGF-β signaling pathway is able to modulate miRNA gene expression at a post-transcriptional level and *vice versa*, as miRNAs can also regulate TGF-β signaling pathway [125]. As miRNAs may regulate several biological processes that are common among different pathologies, and they interfere with TGF-β signaling, it is not strange that some miRNAs are implicated in both renal and cardiac fibrosis.

The deep study of such miRNAs may allow a better understanding of the common pathophysiological mechanisms involved in both pathologies. Thus, the knowledge about the expression pattern of these common miRNAs and the respective signaling pathways that they regulate could provide more information about the underlying pathophysiological processes that correlate CKD and CVD, leading to the discovery of new therapeutic targets that could limit the development of both diseases and attenuate their progression.

2.3.4 - MicroRNAs as Therapeutic Targets

MiRNAs regulate multiple complex biological processes throughout the modulation of signaling pathways and the aberrant miRNA expression has been correlated with a variety of diseases. Recently, many studies have shown that the manipulation of miRNA expression may help with disease treatment, becoming a new class of therapeutic tools [126], [127]. However, there are some steps that must be followed so that a miRNA can be a therapeutic target, such as the identification of [127]:

1. MiRNA's signature.
2. MiRNA's mechanism of action.
3. MiRNA's applicability by RNA interference (RNAi).
4. MiRNA's delivery.
5. MiRNA's form *in vivo*.

MiRNAs are great candidates for therapeutic targets but, in order to use them as therapeutic tools, the abovementioned criteria need to be known and understood.

First, it is important to know what the miRNA signature is. This signature is a specific characteristic of a certain miRNA that allows its identification, and it usually corresponds to its expression pattern, which may be dysregulated in a setting of a disease, being upregulated

or downregulated. The use of miRNAs as therapeutic targets consists of restoring their expression to normal levels using antisense miRNAs, also called antagomiRs or miRNA inhibitors, to decrease the expression of upregulated miRNAs or agomiRs, also called miRNAs mimics, to increase the expression of downregulated miRNAs [127], [128].

In order to produce miRNA-based therapeutic targets, it is important to understand the different mechanisms of action by which miRNAs regulate the expression of different genes in different tissues and conditions. Thus, miRNAs may regulate gene expression by binding to the 3' UTR of the mRNA target sequence to either repress translation or promote mRNA degradation through deadenylation and subsequent decapping. Furthermore, miRNAs may bind to other mRNA sites including to the 5' UTR and coding regions to silence gene expression and within promoter regions to induce DNA transcription [63], [129]. This allows the manipulation of miRNAs, therefore knowing the mechanism by which they act in a certain condition allows a personalized modification of the gene expression to correct abnormalities.

Another regulatory pathway involves RNAis, that are biological molecules that silence gene expression by acting at a post-transcriptional level. MiRNAs are capable of silencing gene expression via RISC, which incorporates the miRNA guide strand and Ago proteins, which drives them to their target mRNA to which it binds by complementary sequence [63]. However, this complementary is only near-perfect, so Ago proteins need the participation of GW182 proteins that possess silencing activity. Thus, the formation of the Ago-GW182 complex allows to silence mRNA targets by repressing translation and contributing to mRNA degradation [130], [131].

Although miRNAs are small molecules, little or no miRNA can diffuse through the tissues due to their low permeability. In addition, miRNA instability in the blood, due to the presence of ribonucleases, presents an issue to deliver them safely and effectively as therapeutic targets. Hence, to overcome these problems, several delivery systems have been developed to offer different properties to enhance miRNA therapeutics that can be classified into viral vectors and non-viral carriers [132], [133].

Viral vectors are modified virus that allow the delivery and constant expression of genetic information, either miRNAs or antagomiRs. They include lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses and are usually used to provide a more efficient gene transfection and to promote a sustained miRNA expression [134]. These delivery systems can carry foreign genetic material and infect host cells by penetrating the nucleus membrane in order to transfer the miRNAs sequences into the host genome and guarantee a persistent and stable gene expression. Despite of all these advantages, using viral vectors have some drawbacks, such as its immunogenicity and cytotoxicity and thus, non-viral vectors emerged to overcome these issues [133]–[135].

Non-viral carriers include lipid-based, polymer-based and inorganic delivery systems that despite of having a decreased delivery efficiency, are much safer than the viral vectors. Lipid-based delivery systems use electronically charged liposomes with a hydrophilic and a hydrophobic part to encapsulate the miRNA gene [132]–[134]. Polymer-based delivery systems may use polyethyleneimine (PEI), poly(lactide-co-glycolic acid) (PLGA), chitosan and poly(aminodoamine) (PAMAMs) dendrimers. These are biocompatible systems with electronic nature that encapsulate the gene and carries it to the host cells. Some of them are constituted by hydrophobic and hydrophilic parts to increase the affinity to cell membrane, allowing a more efficient transfection of the gene [133], [134], [136]. Lastly, inorganic carriers include nanoparticles usually fused with gold, iron oxide (Fe_3O_4) and silica particles to promote stronger interactions with the miRNA, providing a more efficient transfection [133], [134].

The identification of the miRNA form *in vivo* is done by several techniques that help understand the process of gene regulation and their enrollment in pathological mechanisms by providing a real-time tracking of the miRNA dynamics. There are several methods to determine the miRNA expression levels including northern blotting, microarray analysis, RNA next-generation sequence and qRT-PCR, but they are expensive, time-consuming and do not enable the analysis of miRNA dynamics, making them unsuitable for *in vivo* detection. Therefore, to overcome these issues, several imaging techniques have been emerging to allow a sensitive and non-invasive quantification of miRNA expression including bioluminescence imaging (BLI), fluorescence imaging and molecular beacon-based systems [137]. BLI uses photoenzymes that penetrate the tissue and emit quantifiable photons when there is an interaction between the miRNA and its mRNA target sequences. This technique is very sensitive given that there is no background bioluminescence signal [137], [138]. The fluorescence imaging is an ultrasensitive technique that does not require product amplification. This technique uses fluorophores of different colors that bind to specific miRNAs, allowing the tracking of several miRNAs at the same time, which provides more information about their involvement in several biological processes [137], [139]. The molecular beacon connects the fluorophore to a quencher by stem-loop complementary and when there is hybridization, the stem sequences separate by the fluorophore and the quencher, promoting the recovery of the fluorescence signal. This is a low-cost technique when comparing to the qRT-PCR and is more efficient to monitor miRNA expression levels [137], [140].

Overall, the understanding of all these parameters could elucidate the miRNA dynamics during a pathological process, contributing to the identification of novel biomarkers that could act as therapeutic targets with potential clinical applications.

Chapter 3

Objectives

The proposed research project aims to investigate the expression of a selected panel of circulating miRNAs in pre-dialysis CKD patients and explore its association with inflammation and interstitial fibrosis in renal biopsies, as well as to investigate their potential as biomarkers and as novel therapeutic targets, looking forward to obtain transferable results to the clinical field. To this end, the following specific aims were established:

1. Comparative analysis of circulating miRNA expression between CKD patients and HD.
2. Correlation between circulating miRNA expression and clinical data from CKD patients namely:
    a. Renal Fibrosis;
    b. Estimated Glomerular Filtration Rate;
    c. Creatinine, urea, albumin and CRP levels.
3. Correlation between circulating miRNA expression and CVD, among other comorbidities, in CKD patients.
4. Correlation between circulating miRNA expression and CKD progression in CKD patients.

Chapter 4

Materials and Methods

4.1 – Study Population

This study was carried out using blood samples from two different cohorts that were collected from CKD patients enrolled from the outpatient's clinic of Nephrology Department of CHUSJ. This first group of patients consists of a prospective cohort of 44 CKD patients called "discovery cohort", and the second group is composed of 56 CKD patients of a retrospective cohort called "validation cohort". Plasma samples from 58 HD to which creatinine levels were evaluated and found to be within normal values were used as controls.

Patients under 18 years old and/or with history of urinary infections within the last 3 months, or with cancer diagnosis were excluded from the discovery cohort. Patients suffering from acute kidney disease, active or recent infections (<1 week), known psychiatric disturbances and recent hospital admission (<2 weeks) were excluded from the validation cohort. All participants were informed about the study and after signing an informed consent, blood samples were collected.

This research was performed following the Declaration of Helsinki (2008) of World Medical Association and was approved by the Ethics Committee for Health and Local Institutional Review Board of CHUSJ.

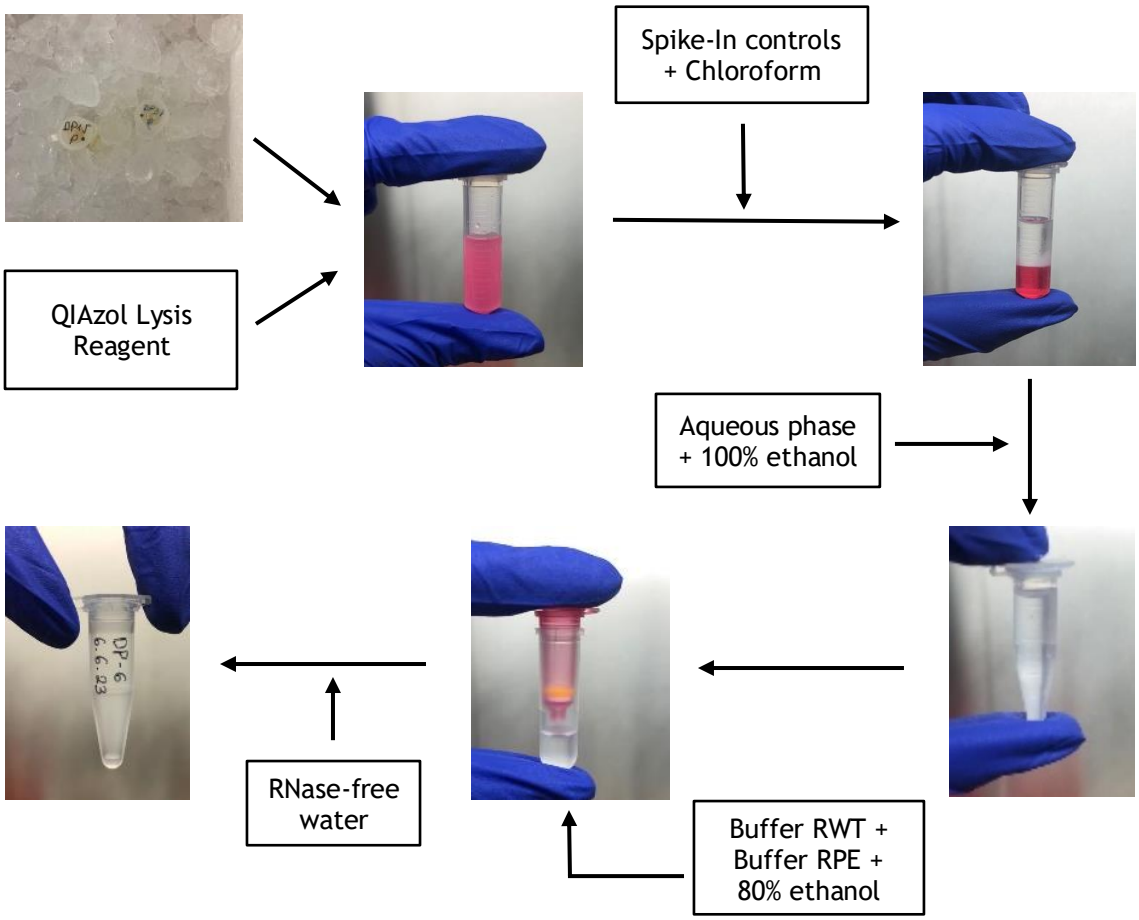
4.2 – Sample Collection of Human Plasma

To obtain the plasma, blood samples from HD and CKD patients were collected in EDTA-blood collection tubes and centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant (plasma) was collected in aliquots of 1 mL in RNase-free eppendorfs and stored at -80°C until further use. Plasma samples that suffer from hemolysis were discarded from the study.

4.3 – RNA Isolation and Purification

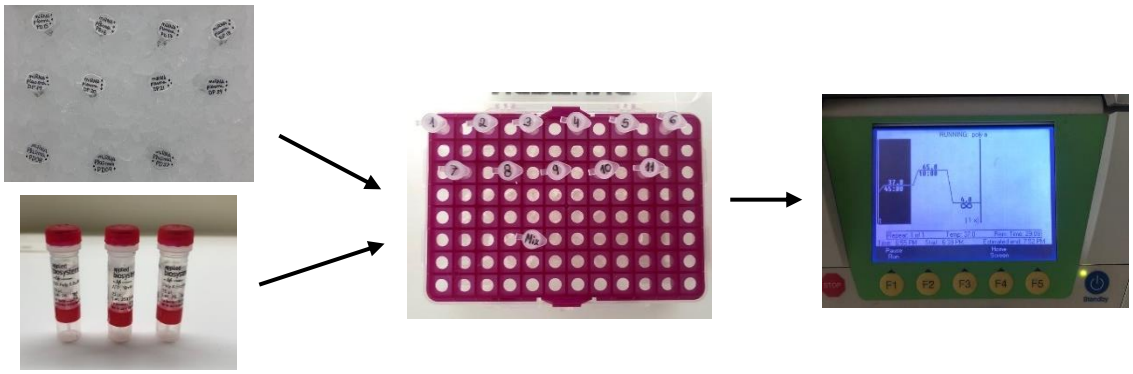
RNA isolation was performed with a miRNeasy Serum/Plasma kit (ref: 50921184, Qiagen, Hilden, Germany) and a QIAzol Lysis Reagent kit (ref: 79306, Qiagen, Hilden, Germany). Plasma samples were prepared according to the manufacturer's instructions. Briefly, 1.0 mL of QIAzol

Lysis Reagent was added to 200 µL of plasma sample and mixed by pipetting up and down, followed by incubating at room temperature (15-25°C) for 5 minutes. After adding 2.0 µL of miRNeasy Serum/Plasma Spike-In controls (cel-miR-39-3p and cel-miR-54-3p [Assay ID: 478293_mir and 478410_mir, respectively]), 200 µL of chloroform were added to each sample and shaken vigorously for 15 seconds, followed by a 2-3 minutes incubation at room temperature. Then, the solution was centrifuged at 12000*g* for 15 minutes at 4°C, and the upper aqueous phase was transferred to a new collection tube and mixed with 900 µL of 100% ethanol by shaking. Subsequently, 700 µL of sample were pipetted into a RNeasy MinElute spin column placed in a 2 mL collection tube and centrifuged at 8000*g* for 15 seconds at room temperature followed by discarding of the flow-through. This step was repeated using the remainder of the sample. After this, 700 µL of Buffer RWT were added to the column and centrifuged at 8000*g* for 15 seconds with subsequent discard of the flow-through, and then, 500 µL of Buffer RPE were added to the column and centrifuged following the same parameters. Afterwards, 500 µL of 80% ethanol were added to the mix and centrifuged at 8000*g* for 2 minutes with subsequent flow-through and tube collection discard. Then, the RNeasy MinElute spin column was placed in a new 2 mL collection tube and centrifugated for 5 minutes at full speed (~15000*g*) with the lid open. Finally, after discarding the flow-through and the collection tube, the spin column was placed in a new 1.5 mL collection tube, and 14 µL of RNase-free water were directly added to the center of the column membrane to centrifuge at full speed for 1 minute to elute the RNA.



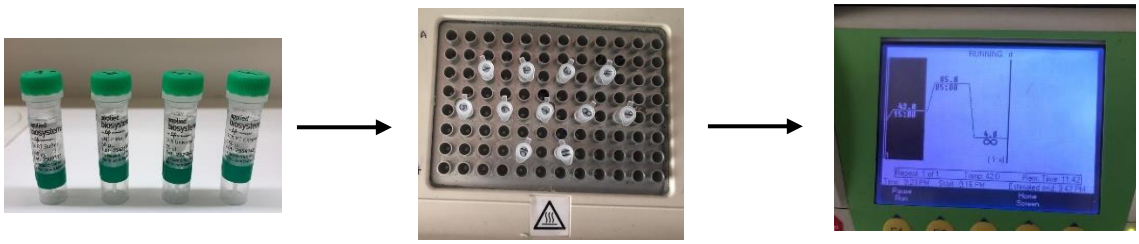
4.4 – Reverse Transcription

After RNA isolation, the reverse transcription reaction was carried out with a TaqMan Advanced miRNA cDNA synthesis kit (ref: A28007, Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. This reaction begins with the poly(A) tailing reaction where 0.5 µL of 10x Poly(A) Buffer, 0.5 µL of ATP, 0.3 µL of Poly(A) Enzyme and 1.7 µL of RNase-free water were added to 2.0 µL of each RNA sample. After that, the samples were incubated in a thermocycle (MyCycler thermal cycler, Bio-rad, Hercules, CA, USA) for 45 minutes at 37°C followed by a second incubation for 10 minutes at 65°C.



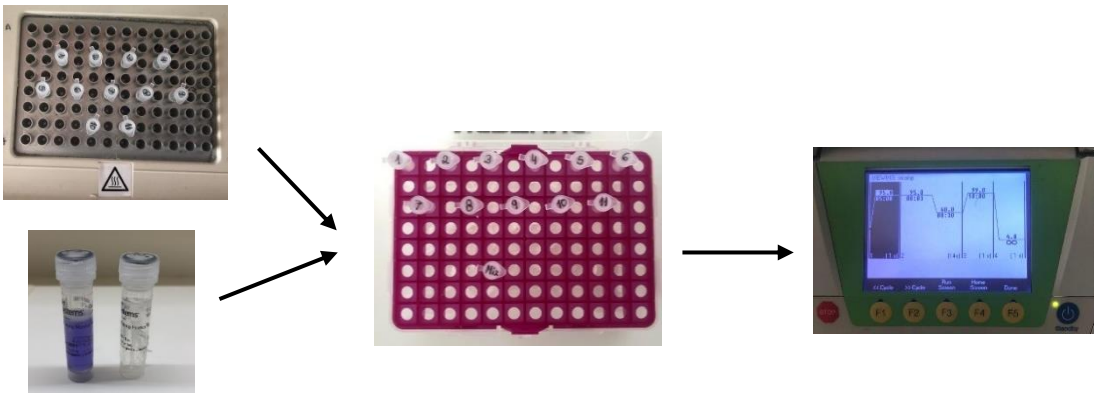
After the poly(A) tailing reaction was done, the adaptor ligation reaction took place and each sample was mixed with 3.0 µL of 5x Ligase Buffer, 4.5 µL of 50% PEG 8000, 0.6 µL of 25x Ligation Adaptor, 1.5 µL of RNA Ligase and 0.4 µL RNase-free water followed by incubation in the thermocycle for 60 minutes at 16°C.

Next, the reverse transcription reaction was performed by adding 6.0 µL of 5x RT Buffer, 1.2 µL of dNTP Mix, 1.5 µL of 20x Universal RT Primer, 3.0 µL of 10x RT Enzyme Mix and 3.3 µL of RNase-free water to each sample and incubated in the thermocycle for 15 minutes at 42°C followed by 5 minutes at 85°C.



Finally, after the reverse transcription reaction was done, a pre-amplification of cDNA reaction was performed. For that, 5.0 µL of each cDNA sample from the previous reaction were transferred into a new eppendorf and mixed with 25 µL of 2x miR-Amp Master Mix, 2.5 µL of 20x miR-Amp Primer Mix and 17.5 µL of RNase-free water. After this, the samples were

incubated for 5 minutes at 95°C followed by 14 cycles of 3 seconds at 95°C, of 30 seconds at 60°C and 1 cycle for 10 minutes at 99°C.

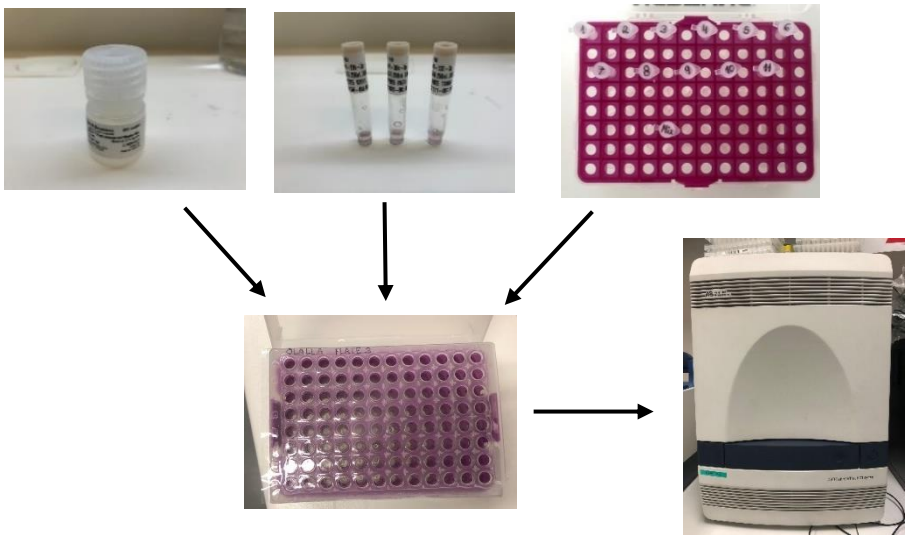


After the reaction was finished, the solution was diluted with 50 µL of RNase-free water and stored at -20°C until further use.

It is important to note that all samples were mixed by vortex and subjected to a spin before each incubation in the thermocycler.

4.5 – Real-Time qRT-PCR

The real-time qRT-PCR reaction was executed using a TaqMan Fast Advanced Master Mix (ref: 4444557, Applied Biosystems, Carlsbad, CA, USA) along with TaqMan Advanced microRNA Assays specific for each miRNA (ref: A25576, Applied Biosystems, Carlsbad, CA, USA). For this purpose, 5.0 µL of TaqMan Fast Advanced Master Mix, 0.5 µL of TaqMan Advanced microRNA Assays and 2.0 µL of RNase-free water were added to each well of a 96 well-plate along with 2.5 µL of each cDNA sample. Each sample was amplified by triplicate and inter-plate controls were added to each plate to assure the absence of variations between plates running. After, the plate was sealed, it was vortexed and centrifuged at 2000 rpm for 2 minutes at 4°C, followed by incubation in a 7500 Fast Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA, USA) for 20 seconds at 95°C, followed by 40 cycles of 3 seconds at 95°C, ending with an incubation at 60°C for 30 seconds.



It is important to mention that cel-miR-39-3p and cel-miR-54-3p genes were used as exogenous controls. After the quantification of the samples, the relative miRNA expression was calculated using the ΔCq method according to the MIQE guidelines.

4.6 – Statistical Analysis

The obtained results were analyzed using the GraphPad Prism v8.1.2 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics v26 (IBM, Manhattan, NY, USA) software. Shapiro-Wilk and Kolmogorov-Smirnov normality tests were used to study the distribution of the data. When data followed a normal distribution, the unpaired t test was applied to study the differences between groups. Non-parametric data were analyzed by Mann-Whitney test to assess significant differences between two groups, and Kruskal Wallis test was used to evaluate variables categorized in more than two groups. Association between numeric variables were analyzed studying Pearson and Spearman's correlation coefficients to study normal and non-normal data, respectively. Statistical significance was considered when p-value < 0.05.

Chapter 5

Results

5.1 – Cohort Analysis

This study entails plasma samples from two cohorts of CKD patients that were established at different time points and from which the so far available clinical data is different. Cohort I was established in 2020-2022 and includes 44 CKD patients that have underwent kidney biopsy in the Nephrology Department of CHUSJ. In this cohort, the detailed clinical data is still missing and only epidemiological data, CKD stage and the percentage of kidney tissue fibrosis is available. Cohort II was established in 2018 and comprises 56 CKD patients from Dra. Ana Cerqueira's outpatients' clinics which have a detailed clinical evaluation, namely cardiovascular outcomes and 5 years follow up of clinical data.

Plasma samples from 58 HD in which creatinine values were measured and considered "normal" (0.63 – 1.16 mg/dL for men and 0.48 – 0.93 mg/dL for women, data not shown) [141] were used as controls.

Given the different time points at which samples were collected and the different storage time, Cohorts I and II were analyzed separately and served as validation for the analysis that could be performed in both, namely for the comparison of miRNA expression between CKD patients and HD. As the sample availability was much larger in Cohort I than in Cohort II, the initial analysis aiming the screening of miRNA differences between CKD and HD samples was performed in Cohort I and the identified differences were validated in Cohort II. Considering the investigation of correlations between miRNAs expression and clinical data, Cohort II was used due to the lack of clinical data from Cohort I and also because of the fact that Cohort II had a longer follow up time (~60 months), therefore allowing the investigation of the relevance of miRNA as predictors for disease progression or comorbidities.

The patient's demographic, clinical and etiologic characteristics from both cohorts are represented in Table 5.1 and in Figure 5.1.

Table 5.1 – Patient's demographic and clinical characteristics of both cohorts. HD: healthy blood donors.

	Cohort I	Cohort II	Control (HD)	*p*-value Cohort I vs Cohort II	*p*-value Cohort I vs HD	*p*-value Cohort II vs HD
Demographic Characteristics						
Age (years)	49.56 ± 14.32	57.00 ± 13.89	38.24 ± 10.60	0.026	0.002	0.090
Gender ratio (male:female)	19:25	29:24	18:20			
Clinical Characteristics						
eGFR (mL/min)	No data	59.89 ± 40.81				
Creatinine (mg/dL)		1.87 ± 1.44				
Urea (mg/dL)		80.79 ± 51.75				
Albumin (g/L)		39.21 ± 6.57				
C-Reactive Protein (mg/L)		8.24 ± 17.10				

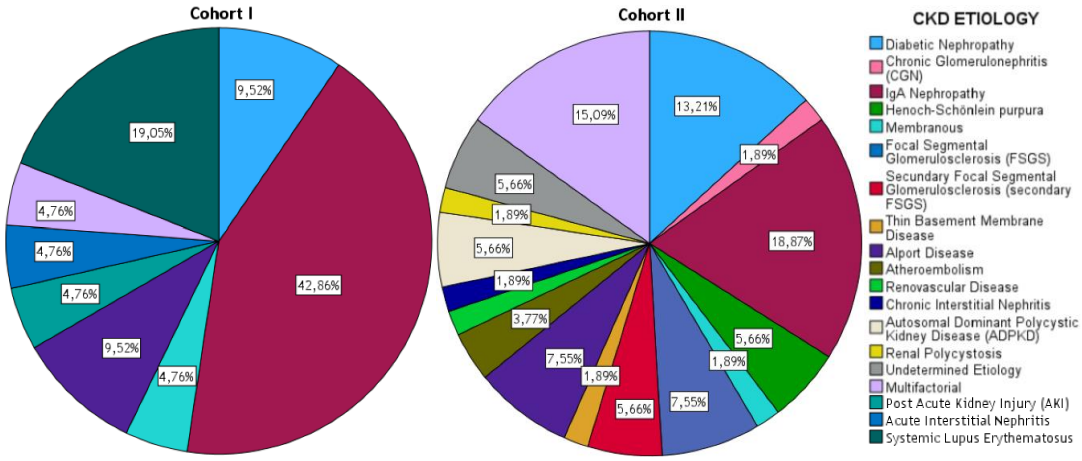


Figure 5.1 - Patient's characteristics according to disease etiology from both cohorts.

The mean age of the patients in Cohort I was 49.56 ± 14.32 years and in Cohort II was 57.00 ± 13.89 years. CKD patients from Cohort I were found to be significantly older than HD (p = 0.002), and significantly younger than CKD patients from Cohort II (p = 0.026). No significant age differences were found between Cohort II and HD (p = 0.090). Furthermore, concerning disease etiology diagnosis, 42.86% and 18.87% of the patients presented IgA nephropathy in Cohort I and Cohort II, respectively, being this the most common CKD etiology among the participants.

5.2 – Comparative Analysis of Circulating MicroRNAs Expression between Chronic Kidney Disease Patients and Healthy Blood Donors

The qRT-PCR analysis of miRNAs expression showed expression of miRNAs miR-21-5p, miR-29b-3p, miR-30c-5p, miR-132-3p, miR-146a-5p, miR-199a-5p and miR-324-3p in all samples (Cohort I, Cohort II and HD). MiR-203a-3p and let-7c-5p were not detected in any sample even using undiluted samples (data not shown).

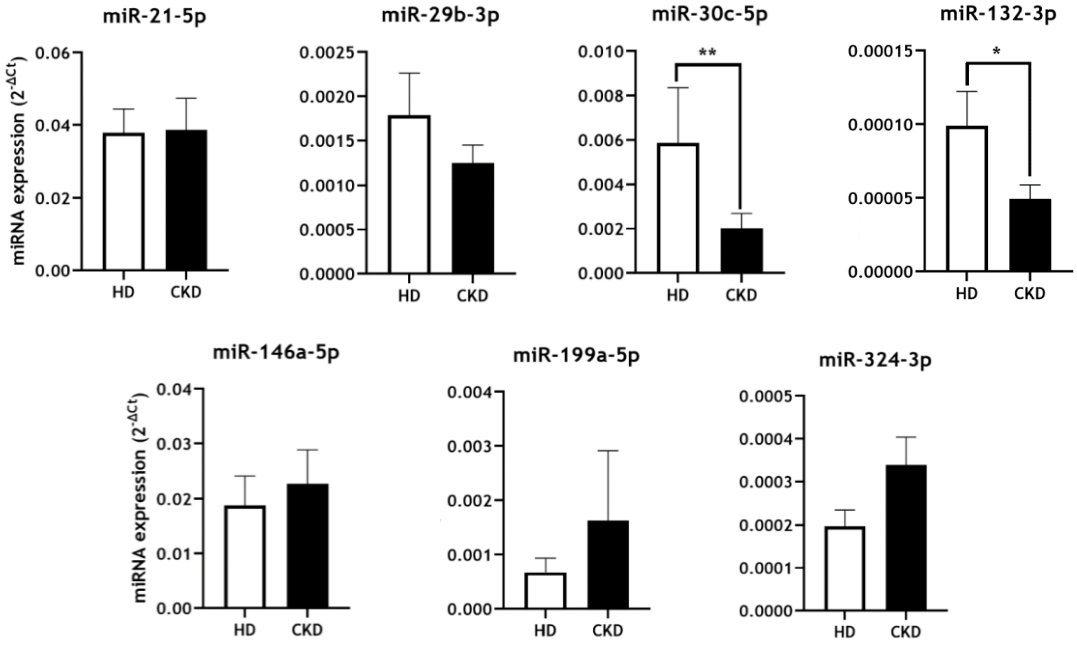


Figure 5.2 - Comparative analysis of circulating miRNAs expression between CKD patients (cohort I, n = 44) and HD (n = 38). Graphs represent the mean ± SEM (* $p < 0.05$ and ** $p < 0.01$). CKD: chronic kidney disease; HD: healthy blood donors; miRNA: microRNA; SEM: standard error of the mean.

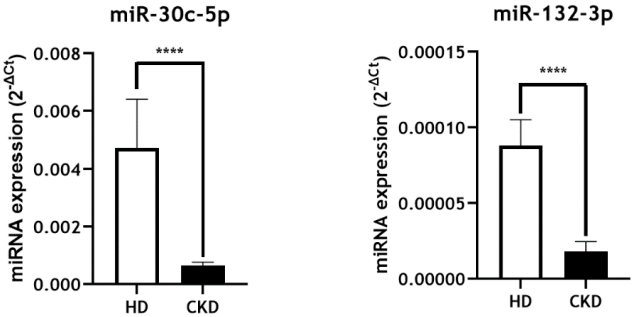


Figure 5.3 - Expression of miR-30c-5p and miR-132-3p in plasma samples of HD (n = 58) and CKD patients (cohort II, n = 56). Graphs show the mean ± SEM (**** $p < 0.0001$). CKD: chronic kidney disease; HD: healthy blood donors; miRNA: microRNA; SEM: standard error of the mean.

Results show that miR-30c-5p and miR-132-3p were significantly downregulated in CKD patients from Cohort I ($p < 0.01$ and $p < 0.05$, respectively) (Figure 5.2). These results were further validated in Cohort II (Figure 5.3). MiR-29b-3p levels show a tendency to decrease in disease condition, and miR-146a-5p, miR-199a-5p and miR-324-3p tend to increase their expression in CKD patients, but no statistical significance was found in these cases. No

differences were observed in miR-21-5p expression between HD and CKD patients (Figure 5.2). The results in Cohort I were not affected by gender or age. In Cohort II miRNA expression was significantly correlated with age (data not shown) and, therefore, a linear regression adjustment of the *p*-value by age was performed. After adjustment, the statistical differences were maintained for miR-30c-5p and miR-132-3p expression between HD and CKD.

5.2.1 – Specificity and Sensibility of Circulating MicroRNAs in Differentiating Chronic Kidney Disease

Considering the statistically significant differences in miR-30c-5p and miR-132-3p between CKD patients and HD in both cohorts, a ROC analysis of these miRNAs was performed to understand their potential as diagnostic biomarkers for CKD.

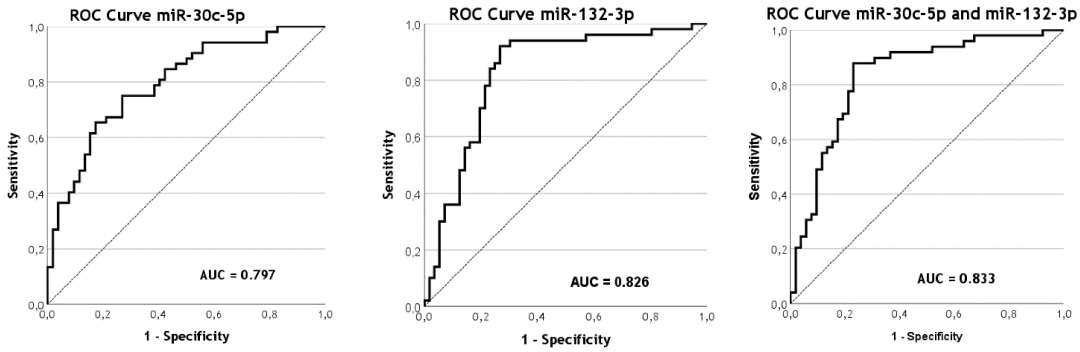


Figure 5.4 - ROC curve of miR-30c-5p, miR-132-3p and of a combinatory panel of both miRNAs in plasma samples of CKD patients (n = 56) and HD (n = 58). AUC: area under the curve; CKD: chronic kidney disease; HD: healthy blood donors; ROC: receiver operating characteristic.

The ROC analysis of miR-30c-5p expression showed 78.8% of sensitivity and 60.4% of specificity in distinguishing CKD patients from HD, corresponding to an AUC of 0.797. The miR-132-3p expression had a sensitivity of 82% and a specificity of 76.8% with an AUC of 0.826. Combining both miRNAs, the ROC analysis showed an AUC of 0.833, which is higher in comparison to each miRNA separately and corresponds to a sensitivity of 83.7% and a specificity of 76.9%, providing a more efficient diagnosis of CKD.

5.3 – Correlation between Circulating MicroRNAs Expression and Clinical Data in Chronic Kidney Disease Patients

5.3.1 - Circulating MicroRNAs Expression and Renal Fibrosis

For the analysis of correlations between miRNA expression levels and the percentage of renal tissue fibrosis, Cohort I was used.

Table 5.2 - Correlation between miRNA expression levels and fibrosis percentage in CKD patients (cohort I, n = 44). Spearman's Rho correlation coefficient (ρ) and *p*-value are shown for each miRNA (* $p < 0.05$ and ** $p < 0.01$). CKD: chronic kidney disease; miRNA: microRNA.

miRNA	ρ	*p*-value
miR-21-5p	-0.321	**0.041***
miR-29b-3p	-0.347	**0.021***
miR-30c-5p	-0.411	**0.007****
miR-132-3p	-0.420	**0.006****
miR-146a-5p	-0.292	0.054
miR-199a-5p	-0.064	0.735
miR-324-3p	-0.351	**0.024***

Results show that miR-21-5p, miR-29b-3p, miR-30c-5p, miR-132-3p and miR-324-3p expression levels were negatively correlated with renal fibrosis. MiR-146a-5p presented a borderline positive correlation ($p = 0.054$) (Table 5.2).

Aiming for a better discrimination of the expression of miRNAs between the different fibrotic states, the kidney biopsies were classified into low (+), moderate (++) or severe (+++) (classification performed by the Medical Pathologist of CHUSJ, Dr. Roberto Silva). Group + is composed of 22 CKD patients that present less than 30% of fibrotic tissue, group ++ is constituted by 18 CKD patients who have between 30% and 69% of fibrotic kidney tissue and only 4 CKD patients were classified in the group +++, which takes into account those who present more than 69% of fibrotic tissue.

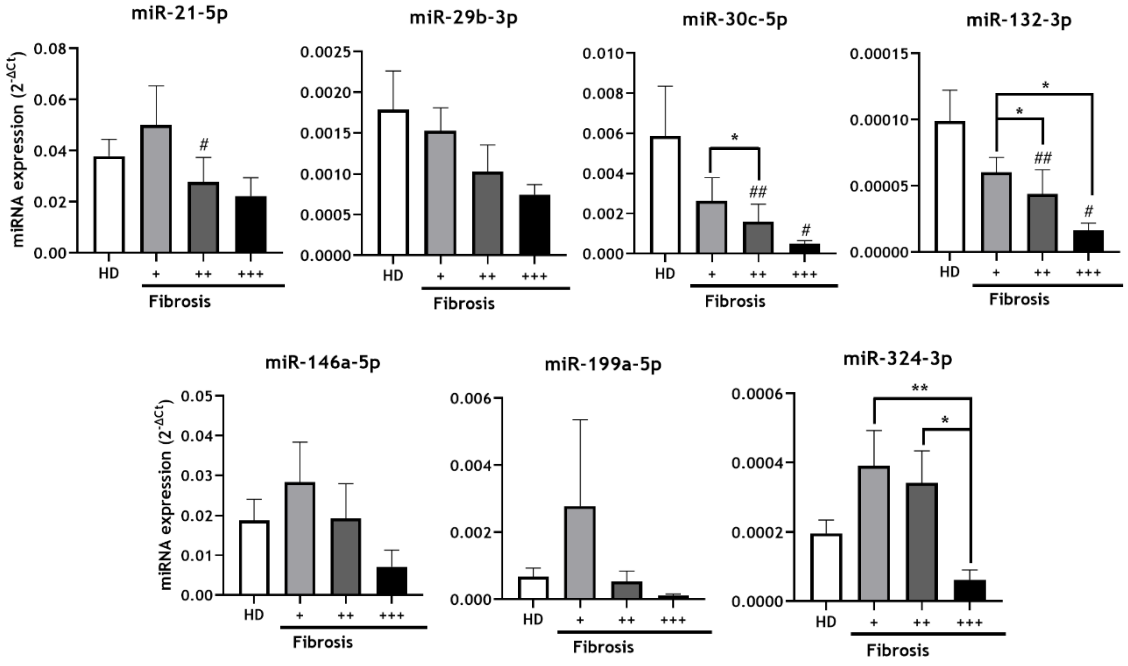


Figure 5.5 - Differential expression of plasmatic miRNAs according to the fibrotic percentage of kidney biopsies of CKD patients, comparison with HD (n = 38). "+": low fibrosis (n = 22), "++": moderate fibrosis (n = 18), "+++": severe fibrosis (n = 4). Graphs show the mean ± SEM (* $p < 0.05$ and ** $p < 0.01$, # $p < 0.05$ and ## $p < 0.01$ regarding HD). CKD: chronic kidney disease; HD: healthy blood donors; miRNA: microRNA; SEM: standard error of the mean.

Despite a tendency was observed in some miRNAs, results revealed no significant differences in miRNAs expression between CKD patients with low fibrosis and HD, and the impact of kidney tissue fibrosis in miRNAs expression was observed only in more severe states. In fact, miR-21-5p, miR-30c-5p and miR-132-3p were shown to be significantly decreased in moderate and severe fibrosis states. MiRNAs miR-29b-3p, miR-146a-5p and miR-199a-5p do not show significant differences in their expression among the different fibrotic states. MiR-21-5p was significantly downregulated in CKD patients with moderate fibrosis ($p < 0.01$), while miR-30c-5p and miR-132-3p show a significant decrease from low to severe fibrotic states ($p < 0.05$). MiR-324-3p is significantly downregulated only in CKD patients with severe fibrosis ($p < 0.01$ and $p < 0.05$ for comparison with low and moderate fibrosis, respectively) (Figure 5.5).

5.3.2 – Circulating MicroRNAs Expression and other Clinical Parameters

The correlation between miRNA expression and the eGFR was assessed in plasma samples from 56 CKD patients (Cohort II).

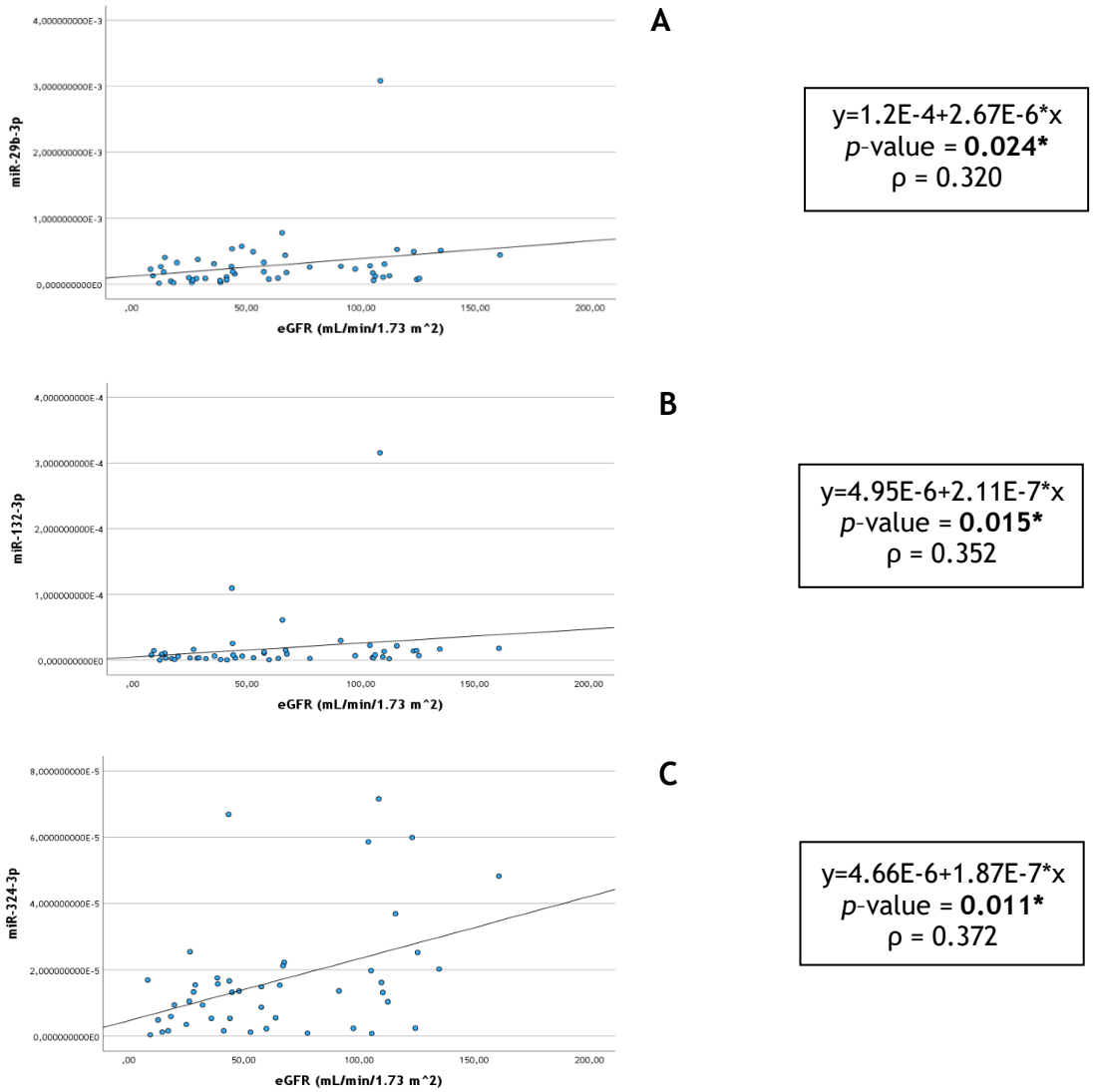


Figure 5.6 - Scatter plot showing the correlation between the expression of (A) miR-29b-3p, (B) miR-132-3p and (C) miR-324-3p and eGFR in plasma samples of CKD patients (n = 56) (* $p < 0.05$). CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate.

Results show that miR-29b-3p, miR-132-3p and miR-324-3p have a significant positive correlation with eGFR ($p < 0.05$) (Figure 5.6).

Considering the fact that 60 mL/min/1.73 m^2 is a threshold used in the clinics to differentiate between a still preserved kidney function from a malfunctioning organ, miRNAs expression was analyzed considering the two groups, i.e., in patients who present eGFR values lower and higher than 60 mL/min/1.73 m^2 (Figure 5.7).

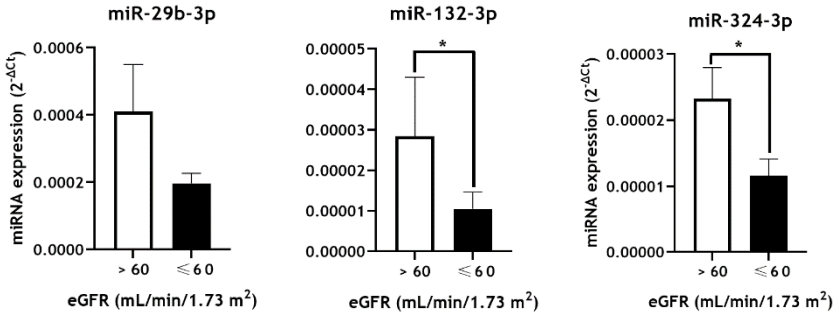


Figure 5.7 - Expression levels of miR-29b-3p, miR-132-3p and miR-324-3p in plasma samples of CKD patients with eGFR levels equal or below (n = 33) and above (n = 35) 60 mL/min/1.73 m^2. Graphs represent the mean ± SEM (* $p < 0.05$). CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate.

Results show that all three miRNAs were decreased in subjects with eGFR equal or lower than 60 mL/min/1.73 m^2, but only miR-132-3p and miR-324-3p expression levels have reached significant statistical differences ($p < 0.05$).

The correlation between circulating miRNA expression levels and clinical data related to renal function including creatinine, urea and albumin was also tested in Cohort II (Table 5.3).

Table 5.3 - Correlation between miRNA expression levels in plasma samples of CKD patients (n = 56) and clinical data related to renal function namely creatinine, urea and albumin. Spearman's Rho correlation coefficient (ρ) and *p*-value are shown for each miRNA (* $p < 0.05$ and ** $p < 0.01$). CKD: chronic kidney disease; miRNA: microRNA.

miRNA		Creatinine	Urea	Albumin
miR-21-5p	ρ	0.019	0.015	0.112
	p-value	0.895	0.921	0.442
miR-29b-3p	ρ	-0.194	-0.250	0.356
	p-value	0.177	0.084	**0.011***
miR-30c-5p	ρ	0.075	0.030	0.273
	p-value	0.609	0.841	0.058
miR-132-3p	ρ	-0.279	-0.261	0.260
	p-value	0.057	0.079	0.078
miR-146a-5p	ρ	-0.170	-0.186	0.116
	p-value	0.243	0.205	0.425
miR-199a-5p	ρ	0.176	0.198	-0.088
	p-value	0.259	0.208	0.577
miR-324-3p	ρ	-0.320	-0.267	0.190
	p-value	**0.030***	0.076	0.205

Results showed that miR-29b-3p was significantly and positively correlated with albumin levels ($p < 0.05$) and miR-324-3p was significantly and negatively correlated with creatinine

levels ($p < 0.05$). MiR-30c-5p levels showed a tendency to correlate positively with creatinine, urea and albumin (Table 5.3). Mir-29b-3p, miR-132-3p, miR-146a-5p and miR-324-3p showed a tendency to correlate negatively with creatinine and urea levels and positively with the albumin levels. MiR-199a-5p showed the opposite correlations, tending to correlate positively with creatinine and urea values and negatively with albumin levels (Table 5.3).

The correlation between circulating miRNA expression levels and CRP was also tested in Cohort II, but only 32 CKD patients presented information regarding the CRP values (Table 5.4).

Table 5.4 - Correlation between miRNA expression in plasma samples of CKD patients (n = 56) and CRP. Spearman's Rho correlation coefficient (ρ) and *p*-value are shown for each miRNA (* $p < 0.05$ and ** $p < 0.01$). CKD: chronic kidney disease; CRP: C-reactive protein; miRNA: microRNA.

miRNA	ρ	*p*-value
miR-21-5p	-0.121	0.546
miR-29b-3p	-0.269	0.176
miR-30c-5p	-0.040	0.845
miR-132-3p	-0.532	**0.005****
miR-146a-5p	-0.090	0.662
miR-199a-5p	0.001	0.995
miR-324-3p	-0.479	**0.015***

Results showed that only miR-199a-5p had a positive correlation with CRP levels. All other miRNAs presented negative correlations with this parameter. In addition, miR-132-3p and miR-324-3p expression showed a significant negative correlation with CRP values ($p < 0.01$ and $p < 0.05$, respectively).

5.3.3 - Circulating MicroRNAs Expression and Comorbidities

The correlation between miRNA expression levels and the presence of comorbidities including diabetes, hypertension, CVD, macrovascular disease and cerebrovascular disease was evaluated in 56 CKD patients (Cohort II).

Table 5.5 – Differences between miRNA expression levels in plasma samples of CKD patients (n = 56) and several comorbidities namely diabetes, hypertension, CVD, macrovascular disease and cerebrovascular disease. *P*-value is shown for each miRNA (* $p < 0.05$). CKD: chronic kidney disease; CVD: cardiovascular disease; miRNA: microRNA.

miRNA	Diabetes	Hypertension	Cardiovascular disease	Macrovascular disease	Cerebrovascular disease
miR-21-5p	0.087	0.328	0.245	0.334	0.508
miR-29b-3p	0.759	0.065	0.362	0.216	0.720
miR-30c-5p	0.515	0.858	0.864	0.723	0.752
miR-132-3p	0.892	0.572	0.323	0.071	0.788
miR-146a-5p	0.883	0.806	1.000	0.973	0.560
miR-199a-5p	0.078	0.052	**0.041***	0.268	0.399
miR-324-3p	0.131	0.345	0.329	0.134	**0.017***

As observed in Table 5.5, miR-199a-5p and miR-324-3p showed to have reached significant statistical differences in CKD patients with a cardiovascular pathology and in CKD patients that suffer from a cerebrovascular disease, respectively ($p < 0.05$).

5.3.4 – Circulating MicroRNAs Expression and Cardiovascular Outcomes

The relative expression of the selected miRNAs was assessed in plasma samples of 56 CKD patients (Cohort II), that were divided into two groups according to the presence or absence of CVD. Among them, 37 CKD patients did not have any cardiovascular outcomes and 16 CKD patients presented a CVD.

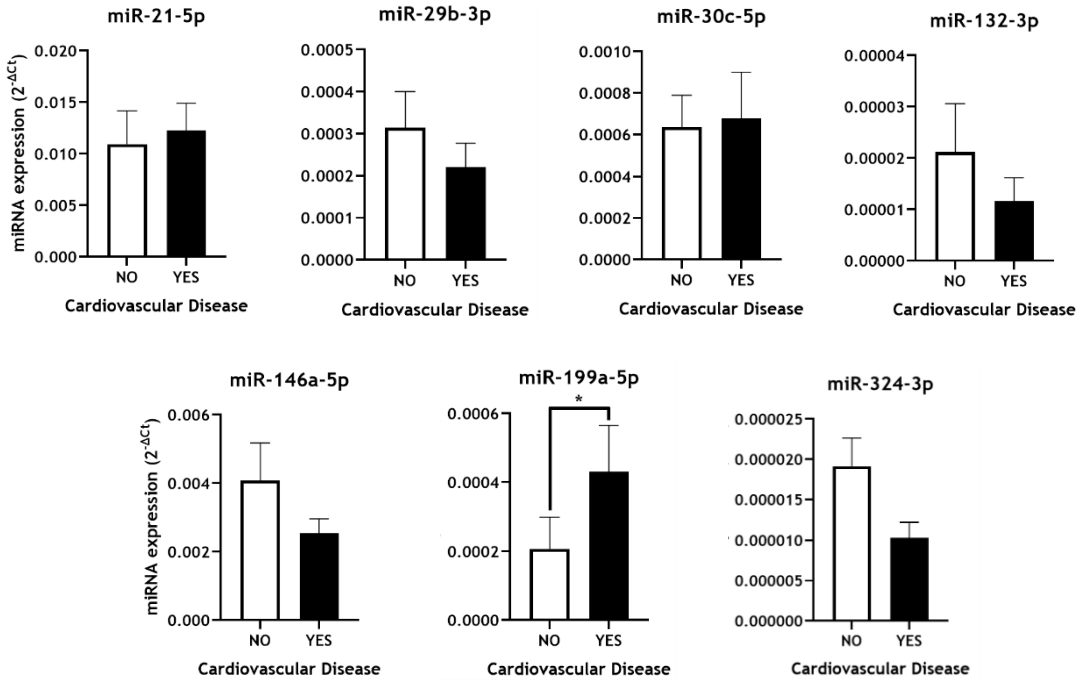


Figure 5.8 - Expression levels of circulating miRNAs in plasma samples of CKD patients with (n = 16) and without (n = 37) CVD. Graphs represent the mean ± SEM (* $p < 0.05$). CKD: chronic kidney disease; CVD: cardiovascular disease; miRNA: microRNA; SEM: standard error of the mean.

Results showed that miR-199a-5p expression is significantly increased in CKD patients that suffer from a CVD ($p < 0.05$). The differences regarding the remaining miRNAs did not reach statistical significance. MiR-21-5p and miR-30c-5p tend to increase their expression in patients with both diseases, whereas miR-29b-3p, miR-132-3p, miR-146a-5p and miR-324-3p showed a tendency to increase in CKD patients that do not have CVD.

5.3.5 – Circulating MicroRNAs Expression and Cerebrovascular Outcomes

The relative expression of miR-324-3p was tested in 56 CKD patients (Cohort II). These patients were classified based on the presence or absence of a cerebrovascular disease and thus divided into two groups, one composed of 5 CKD patients that had a cerebrovascular event and another group with 47 CKD patients without cerebrovascular outcomes.

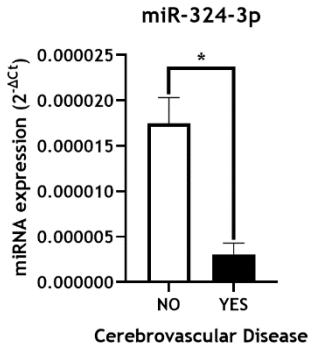


Figure 5.9 - Expression levels of miR-324-3p in plasma samples of CKD patients with (n = 5) or without (n = 47) cerebrovascular disease. Graphs represent the mean ± SEM (* $p < 0.05$). CKD: chronic kidney disease; miRNA: microRNA; SEM: standard error of the mean.

Results showed that miR-324-3p was significantly downregulated in CKD patients with a cerebrovascular disease ($p < 0.05$).

5.3.6 – Circulating MicroRNAs Expression and Progression of Chronic Kidney Disease

The relative expression of circulating miRNAs was assessed in plasma samples of 29 CKD patients (Cohort II). These patients had a 5-year clinical follow up and their stage was registered to evaluate if they would have progressed or not to a worse CKD stage. CKD progression was considered when the patient advanced one or more stages from one appointment to the other.

In Cohort I, 10 CKD patients progressed to a worse CKD stage during the analyzed time period and 19 CKD patients did not progress.

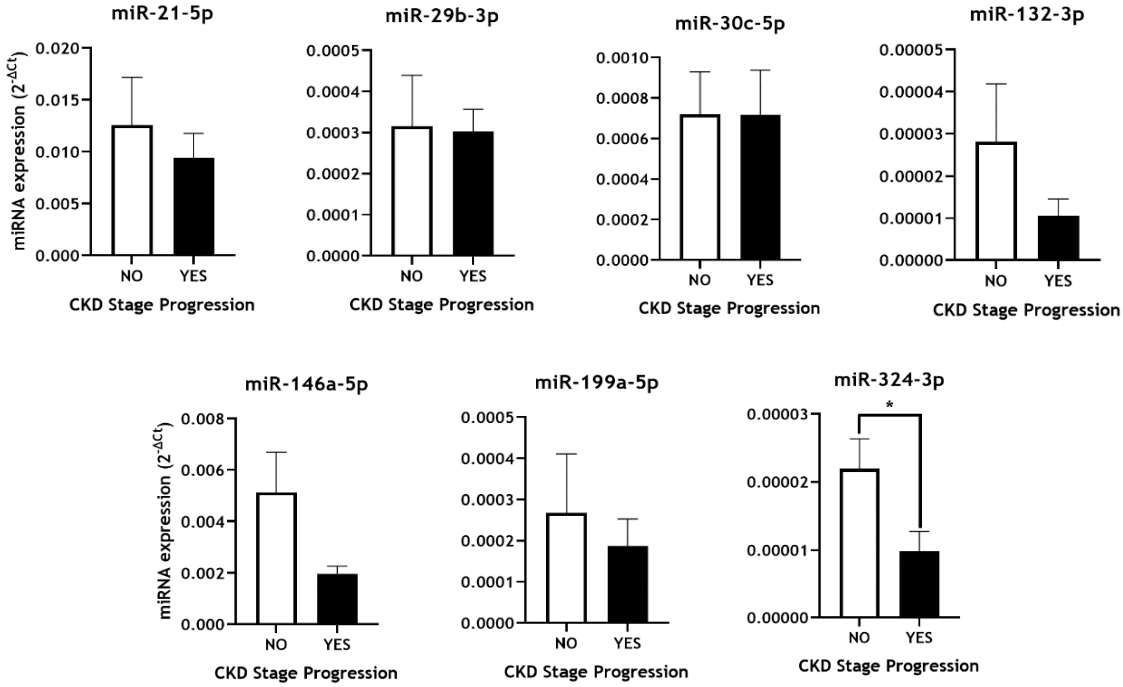


Figure 5.10 - Expression levels of circulating miRNAs in plasma samples of CKD patients with (n = 10) and without (n = 19) CKD stage progression. Graphs represent the mean ± SEM (* $p < 0.05$). CKD: chronic kidney disease; miRNA: microRNA; SEM: standard error of the mean.

Results showed that miR-21-5p, miR-132-3p, miR-146a-5p, miR-199a-5p and miR-324-3p tended to be decreased in patients who progressed into worse CKD stages. Furthermore, miR-324-3p showed to be significantly downregulated in patients with progression to more severe CKD stages ($p < 0.05$).

5.4 – Summary

Table 5.6 - Summary of the results obtained.

MiRNA	CKD vs HD	Fibrosis			eGFR (mL/min/1.73 m^2)	Creatinine (mg/dL)	Albumin (g/L)	C-Reactive Protein (mg/L)	CVD	Cerebrovascular Disease	Progression
		+	++	+++							
miR-21-5p			↓								
miR-29b-3p					+		+				
miR-30c-5p	↓		↓	↓							
miR-132-3p	↓		↓	↓	+			-			
miR-146a-5p											
miR-199a-5p									↑		
miR-324-3p				↓	+	-		-		↓	↓

Summing up, the expression of miR-21-5p showed to be significantly downregulated in CKD patients with moderate fibrosis and the expression of miR-29b-3p showed to be positive correlated with eGFR and albumin levels. In addition, the expression of miR-30c-5p and miR-132-3p was significantly downregulated in CKD patients in comparison to HD, especially in those with moderate and severe fibrosis, and the expression of miR-132-3p was also positive

correlated with eGFR levels, but negatively correlated with CRP values. Furthermore, the expression of miR-199a-5p was found to be significantly upregulated in CKD patients that present a cardiovascular disease. Meanwhile, the expression of miR-324-3p was found to be significantly downregulated in CKD patients with severe fibrosis and positively correlated with eGFR values. It was also found that this miRNA is negative correlated with creatinine and CRP levels, and its expression showed to be significantly downregulated in CKD patients with a cerebrovascular disease and in CKD patients that progressed into a worse CKD stage in a 5-year period.

5.5 – Troubleshooting

In order to optimize the method to study miRNA expression in human samples, an RNA extraction was carried out from an initial group of plasma samples from CKD patients and HD. One of the first steps of this procedure consists of adding chloroform after cell lysis in order to achieve the separation of the sample into layers. Subsequently, after a centrifugation, an upper aqueous phase is obtained that contains RNA, an interphase and an organic phase where DNA, proteins and lipids are present. Initially, there was a drawback at this point of the technique as some samples did not separate into aqueous phase, interphase, and organic phase. To solve this problem, different lysis reagents were tested including QIAzol, TRIzol and EXTRAzol. As some samples continue not to separate, different proportions of lysis reagent-sample were also tested. After the tests, QIAzol lysis reagent in a proportion of 1 mL/200 µL sample were the conditions selected, and some changes in the protocol were made as shake carefully every reagent before their use and shake vigorously after the addition of chloroform instead of vortex the samples. After these improvements, the separation of the aqueous phase was achieved in all the samples.

Chapter 6

Discussion

Over the past few years, CKD has been emerging as a major public health problem and CVD is a major contributor for these patients' death [40]. However, the underlying pathophysiological mechanisms behind the relationship of CKD and CVD are still far from being understood. Recently, miRNAs have been arising as important regulators of gene expression and several have been associated with human pathologies [60], [69]. These molecules are highly stable in extracellular fluids, which allows their detection through non-invasive methods [4], [60], [62], therefore it is of great interest to investigate their role as diagnostic biomarkers for the early detection of CKD and cardiovascular outcomes and as potential therapeutic targets to provide patient-tailored therapies.

In this study the relative expression of 7 miRNAs, which were previously described as being involved in CKD progression and CVD, was analyzed in plasma samples from two different cohorts of CKD patients from CHUSJ. Cohort I entailed samples that were more recently collected (2020-2022), but from which it still lacked many clinical data and Cohort II comprised samples that were collected in 2018 and from which the clinical data was accessed at the time of sample collection and upon 5 years follow up.

Taking into consideration the fact that sample availability from Cohort II was scarce, as starting point, it was decided to perform a comparative analysis of miRNA expression between CKD and control samples (plasma samples from HD) using the plasma samples from Cohort I (that do not have the sample availability constrains of Cohort II). Such analysis showed that miR-30c-5p and miR-132-3p were significantly downregulated in CKD patients. In order to further validate these results, the same analysis was performed using the samples from Cohort II and the same result was observed. Moreover, both the individual and combinatorial ROC analysis of these two miRNAs showed high specificity and sensitivity (76.9% and 83.7%, respectively) for differentiating the CKD condition which is indicative of its single and, most particularly, combinatorial value as potential diagnostic biomarkers for CKD. Previous studies in human kidney tissue and human kidney (HK-2) cells from diabetic nephropathy (DN) patients have demonstrated that the downregulation of miR-30c-5p is capable of enhancing EMT processes via PI3K/AKT signaling pathway, accompanied by increased levels of collagen type I and fibronectin [101], [142]. Such data is in accordance with the findings and support an antifibrotic role of miR-30c-5p in kidney diseases.

In regard to miR-132-3p, Han el al. demonstrated that the upregulation of this miRNA increases the inflammatory markers TNF-α and IL-1ß by activating the NF-κB signaling pathway in HK-2 cells and in a mouse model of acute kidney injury [111]. Another study found that silencing miR-132 results in a reduction in collagen type I expression and in myofibroblast proliferation by TGF-ß signaling pathway, exerting protective effects against kidney fibrosis in an unilateral ureteral obstruction mouse (UUO) model and in mouse cells [108]. Considering the fact that a reduction of miR-132-3p expression in CKD patients was observed, the reported data is in contradiction with the results obtained. Still, there are critical differences among the studies, namely and foremost, the fact that human plasma from CKD patients were used while the former studies report data from animal models and *in vitro* cellular assays, which may not be reproducible of what happens in the human settings. Moreover, as miRNAs often regulate multiple pathways, different intermediates, from the ones identified in the reported assays, may be involved in humans resulting in an overall protective effect of miR-132-3p when assessed in human plasma.

In fact, miR-132-3p has also showed a statistically significant positive correlation with eGFR, which further supports a protective role against CKD, and both miR-132-3p and miR-30c-5p displayed a negative correlation with the percentage of kidney tissue fibrosis, reinforcing a putative antifibrotic contribution in renal disease.

Moreover, miR-21-5p, miR-29b-3p and miR-324-3p were found to have a negative correlation with kidney fibrosis. In order to shed light into the enrollment of these miRNAs in the evolution of kidney fibrosis, its expression was evaluated considering different fibrotic degrees of the kidney tissue. Results showed that miR-29b-3p, miR-30c-5p and miR-132-3p present a trend of continuous decrease from low to severe fibrotic stages, suggestive of their involvement since early stages of tissue fibrosis. For miR-30c-5p and miR-132-3p, a significant downregulation was observed in moderate and severe fibrosis. MiR-21-5p, miR-146a-5p, miR-199a-5p and miR-324-3p do not present any decrease in low and moderate fibrosis being tendentially reduced only in severe fibrotic states (> 69% of fibrosis), which may support a differential role in later CKD disease stages. In fact, in similarity with miR-132-3p, miR-324-3p was also significantly reduced in patients with eGFR lower than 60 mL/min/1.73 m^2, i.e., with compromised renal function in comparison with patients with functional kidneys (eGFR higher than 60 mL/min/1.73 m^2). As it was abovementioned, the upregulation of miR-132-3p increases myofibroblasts proliferation via TGF-ß signaling pathway in UUO mouse model, as well as miR-324-3p as demonstrated by Macconi et al. in rats [108], [121]. Although both miRNAs are correlated with severe fibrotic states, they increase myofibroblast proliferation, an early step of fibrosis, which results in an excessive deposition of ECM compounds contributing to a fibrogenic environment.

The so far available data regarding the role of miR-21-5p and miR-324-3p are, as observed for miR-132-3p, in contradiction with the findings. For miR-324-3p, our evidence of downregulation in CKD is further supported by the identified negative correlation with creatinine levels. CKD progression is characterized by progressive reduction in renal function as a consequent of renal fibrosis, which decreases the kidney's filtration capacity resulting in an accumulation of several waste products including creatinine [143]. In the case of this miRNA, the identified contradiction with the published data may be attributed to the very different experimental settings (animal models *vs* plasma from CKD patients). For miR-21-5p, several evidences, even in human settings, support an upregulation in CKD which the data obtained do not corroborate. Zhong et al. showed models and Bharti et al. demonstrated that the

upregulation of TGF-β1 levels in plasma of IgA nephropathy patients was positively correlated with miR-21-5p expression [77], [83]. Still, a different report from Saejong et al. did not corroborate such finding in whole plasma, having identified an upregulation only in plasmatic exosome [144]. Considering the heterogeneity of the results and the fact that the experimental data in human settings are very scarce, further analysis of miR-21-5p in different CKD cohorts under very well-established protocols are required for the better understanding of its role in CKD.

In this investigation, miR-29b-3p was positively correlated with albumin. The accumulation of albumin in the urine results in albuminuria, which promotes renal inflammation and fibrosis, increasing the progression of CKD stage up to 10-fold [145]. Furthermore, as miR-29b-3p and albumin have a positive correlation, the downregulation of this miRNA promotes the downregulation of albumin, which retards the setting of fibrosis.

In regard to correlation with established comorbidities, at the time of sample collection, only miR-199a-5p and miR-324-3p were enrolled. MiR-199a-5p was positively correlated with CVD and miR-324-3p showed a negative correlation with cerebrovascular disease. Wu et al. found that the upregulation of miR-199a-5p in rat mesangial cells increases renal inflammatory factors, as MCP-1, and fibrotic factors, such as CTGF, by regulating the NF-κB signaling pathway [115]. Another study conducted by Zeng et al. in mouse CFs and in a mouse model of cardiac fibrosis demonstrated that the upregulation of miR-199a-5p increases the expression of collagen type I with consequent decrease of ejection fraction by activating the NF-κB signaling pathway, contributing to cardiac fibrosis [116]. These findings are consistent with what was found in the study and suggest that miR-199a-5p exerts its profibrotic effects by activating the NF-κB signaling pathway. In addition, miR-324-3p showed to be significantly downregulated in CKD patients with cerebrovascular outcomes. Dharap et al. reported that the upregulation of this miRNA in PC12 adherent cells is associated with an increase of the RelA gene, that is known to promote neuronal death after major insults to the nervous system, by activating the NF-κB pathway [146]. However, as it was abovementioned, the upregulation of miR-324-3p mediates kidney fibrosis by activating the TGF-β pathway [121], therefore further studies are needed to investigate the underlying pathways that correlate the existence of cerebrovascular events in CKD.

With the aim of investigating the potential of the analyzed miRNA as prognostic biomarkers for disease progression and cardiovascular outcomes, the clinical data from Cohort II patients in a 5 year follow up was retrieved and analyzed concerning the differential miRNAs expression.

Disease progression was considered in patients that presented the worsening of CKD by at least one stage regardless of the stage at which they were at. Patients that remained in the same CKD stage for the time period in analysis were considered non-progressive. Results showed that miR-324-3p was significantly downregulated in CKD patients with stage progression. As it was found, the downregulation of this miRNA in severe states of fibrosis is correlated with a differential role in later CKD stages, therefore the continuous downregulation of miR-324-3p expression allows the staging of disease progression. So far, nothing regarding the correlation between the expression of miR-324-3p and the progression of CKD stage has been reported. In addition, as it was already mentioned, the upregulation of this miRNA in rats is correlated with the development of renal fibrosis, which do not corroborate the obtained data [121]. Still, the increase of renal fibrosis results in loss of kidney function and progression of CKD, therefore, it is postulated that miR-324-3p may be involved in the regulation of underlying mechanisms of disease progression.

However, this study presents relevant work for the scientific community given that to the best of my knowledge, this is the first human study to investigate the enrolment of miRNAs in kidney-heart crosstalk in CKD patients.

Chapter 7

Conclusion and Future Perspectives

CKD is a major worldwide public health problem characterized by a sustained chronic inflammatory state that evolves to fibrosis of the organ that can ultimately lead to renal failure. This pathology is rapidly arising as one of the most common causes of morbidity and mortality, being intrinsically correlated with the development of CVD in these patients. So far, the pathways underlying the bidirectional relationship between CKD and CVD remain largely unknown. A better understanding of these processes may lead to the development of diagnosis and therapeutic tools, that would represent a major breakthrough in the management of these conditions and have a significant impact in the patient's health. MiRNAs are an emergent new field of research that has been highlighted as novel diagnostic biomarkers and therapeutic targets in other diseases associated with fibrotic processes. Therefore, considering the circulating miRNAs fraction, it is postulated that miRNAs may serve as a bridging factor between CKD and CVD.

The aim of this research was to study the expression of a miRNAs panel and evaluate if they could act as diagnostic biomarkers and therapeutic targets to renal and cardiovascular outcomes in CKD. To perform this investigation, the relative expression of 7 miRNAs was evaluated in CKD patients and compared with CKD patients with cardiovascular outcomes and HD.

The present study identified miR-30c-5p and miR-132-3p as potential diagnostic biomarkers for the early detection of CKD, in particular when combined together, providing more accurate diagnosis of the disease. Furthermore, given their downregulation under fibrotic conditions, the upregulation of these miRNAs may serve as therapeutic targets for striking this pathology. In addition, miR-324-3p showed to be significantly downregulated in worse fibrotic states, which indicates that this miRNA may be involved in later CKD stages and it also showed to be significantly downregulated in patients who progressed to worse CKD stages. Together, it suggests that miR-324-3p may be a biomarker for prognosis of disease progression and also a target for preventing the progression to worse stages. Furthermore, miR-199a-5p was found to be significantly upregulated in CKD patients with cardiovascular outcomes. Several studies have found that this miRNA regulates both renal and cardiac fibrosis by activating the NF-κB signaling pathway. This demonstrates that targeting miR-199a-5p with inhibitors may suppress the activation of this pathway, thus inhibiting or retarding the fibrotic process in both organs.

This research was the first study to correlate CKD with CVD by using human plasma samples of CKD patients, which brings relevant insights regarding the regulatory mechanisms involved in the kidney-heart fibrosis crosstalk in these patients to the scientific community. In this sense, further studies need to be performed to validate these findings. In the future, the continue collection of human plasma samples of CKD patients would bring great advantages and reliability for this study. Furthermore, these miRNAs could be studied *in vitro* in cultured fibroblasts by their inhibition or supplementation into the medium through the transfection of antagomiRs or agomiRs, respectively, to investigate matrix production. Also, the cells could be treated with TGF-β1 to study the miRNA expression under simulated fibrotic conditions. In addition, *in vivo* studies could be performed by using a rat UUO model, a well-known model of renal fibrosis, to study and confirm the metabolic pathways by which miRNAs contribute to the progression of kidney fibrosis.

Attachments

Additional Information

A - Collaborative Works

A.1 – Cohort Establishment

During my Master's work, I have been actively involved in the establishment of several cohorts of plasma samples from CKD patients from CHUSJ. For this aim, I was responsible for contacting the patients, having them signing the informed consent form, receiving the blood samples, separating the plasma and storing the samples at -80°C. Such collaborative work has granted me experience regarding the work within the hospital environment.

A.2 – Collaboration in the Study "The interaction between circulating MicroRNA and Microbiome in Peritoneal Membrane Fibrosis"

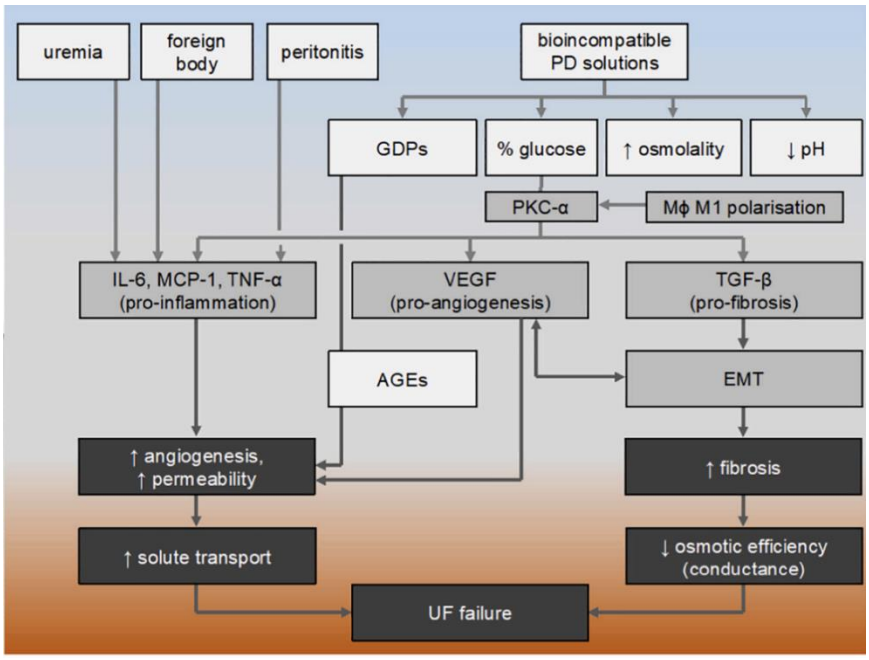


Figure A.1 - Scheme of peritoneum fibrosis: uremia, bacteria and bioincompatible PD solutions damage the mesothelial cells and provoke the release of profibrotic mediators that induce EMT and angiogenesis. Together, they contribute to fibrosis and ultimately, to ultrafiltration failure. Adapted from [147].

B – Oral Presentations in Scientific Meetings

As presenter

Eduarda Carvalho, Olalla Ramil-Gómez, Sara Moura, Ana Cerqueira, Núria Paulo, Roberto Silva, Luís Mendonça, Hugo Diniz, Maria João Sousa, Emílio Gomes, Isabel Brandão, Maria Inês Almeida, Manuel Pestana, Inês Soares Alencastre. "MiRNAs in Kindey-Heart Fibrosis Crosstalk". IJUP23 – *16º Encontro de Investigação Jovem da Universidade do Porto* (May 2023). Portugal.

As co-author

Olalla Ramil-Gómez, **Eduarda Carvalho**, Ana Cerqueira, Núria Paulo, Sara Moura, Roberto Silva, Isabel Brandão, Maria Inês Almeida, Manuel Pestana and Inês Soares Alencastre. "Identification of circulating miRNAs enrolled in chronic kidney disease progression and cardiovascular outcomes". Encontro Renal 23, Sociedade Portuguesa de Nefrologia (November 2023) (accepted).

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C – Publications

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