University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

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2. Clinical Supervisor:

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3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Investigating the role of microRNA in kidney fibrosis

   3.2 Project Lay Summary (copied from application):

   Chronic Kidney Disease (CKD) is a major public health issue, affecting approximately 5% of the population. Preventing progression of CKD towards kidney failure may reduce the need for dialysis and kidney transplantation. Whilst CKD is caused by many conditions, the common problem, once kidney damage has occurred, is kidney scarring. We have demonstrated that small proteins called microRNAs are present in animal models of kidney scarring. We would like to find out if microRNA levels in blood (or urine) correlate with

degree of kidney scarring on biopsy and hope that these represent a method for early detection of kidney scarring.

3.3 Start Date: 9th JUNE 2014 Finish Date: 18 JULY 2014

3.4 Original project aims and objectives (100 words max):

Primary objective:

Do serum or urine miRNA-21 levels correlate with the degree of fibrosis (scarring) at the time of kidney biopsy?

Secondary objectives:

Are serum or urine miRNA-21 levels easy to measure in a large pilot study of patients with CKD undergoing kidney biopsy?

Do different factors predict miRNA-21 levels in patients undergoing kidney transplant biopsy compared to native kidney biopsy?

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

Sample collection

A whole stream urine specimen was collected from patients undergoing kidney biopsy (native or transplant) in Glasgow Western Infirmary with full ethical approval (GN14RE002/14WS0048).

Urinary Exosome isolation

Cells and debris were removed from 2.5 ml urine sample by centrifugation at 2000 x g for 30 minutes. 0.5 ml clarified urine was transferred to a new tube labelled 'urine' and stored. 2ml was transferred to a new tube labelled "exosome' without disturbing the pellet. The same volume of Total Exosome Isolation (from urine) reagent (Life Technologies) was added. This mixture was centrifuged at 10,000 x g for 1 hour at 4^{o}C. The supernatant was discarded and the exosomes are contained in a pellet and resuspended in 40µl of phosphate buffered saline (PBS).

Nanoparticle tracking analysis (NTA)

NTA was carried out using the Nanosight LM10-HS system (NanoSight) on 4 µl of exosomes resuspended in PBS. The Nanosight™ system focuses a laser beam through a suspension of

the particles of interest. These are visualized by light scattering, using a conventional optical microscope aligned normally to the beam axis which collects light scattered from every particle in the field of view. A 20–60 s video records all events for further analysis by NTA software. The Brownian motion of each particle is tracked between frames, ultimately allowing calculation of the size through application of the Stokes-Einstein equation. This measures concentration of exosomes in the sample.

Total RNA isolation

MicroRNA was isolated using the miRNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, 700 µl of QIAzol reagent was added to 2 ml of urine sample. The sample was mixed in a tube before adding 200 µl of chloroform. After mixing vigorously for 15 seconds, the sample was then centrifuged at 12,000 x g for 15 minutes at 4^{o}C. The upper aqueous phase was carefully transferred to a new collection tube, and 900 ml of ethanol containing binding buffer from the kit was added and mixed. The sample was then applied directly to a silica membrane containing column and the RNA was retained and cleaned using buffers provided in the kit. The immobilized cleaned RNA was then eluted from the membrane into a collection tube with a low salt elution buffer or water. The RNA concentration and purity were evaluated by measuring the relative absorbance ratio at 260/280 on a Nanodrop 1000 (Thermo, Wilmington, DE).

Taqman OpenArray System for miRNA expression profiling

MiRNAs were reverse transcribed using the Megaplex RT Primer Pool A and B (Applied Biosystems), which contains RT primers for 754 miRNAs, 3 endogenous controls, and a negative control.

For the open arrays, the RT reaction products were pre-amplified using the Megaplex PreAmp Primers and the TaqMan® PreAmp Master Mix. miRNA expression profile was performed by using Taqman OpenArray Real-Time PCR Master Mix on the Taqman OpenArray Plate. This is loaded onto an 7900HT Fast Real-Time PCR System. Raw Ct values were calculated using the SDS software.

Taqman qRT-PCR

Expression of a subset of miRNAs (miR-21, miR-29c) identified through miRNA expression profiling and a housekeeper miRNA, RNU-48, were quantified using specific Taqman microRNA Assays. RNA was diluted to 2ng/ul and miRNAs reverse transcribed using specific primers (Life Technologies). Following reverse transcription, products were combined with Taqman miRNA Assay and Taqman Universal PCR Master Mix No AmpErase UNG. Subsequently, a qRT-PCR was performed on an Applied Biosystem 7900HT thermocycler.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Patient demographics

We studied a total of 14 subjects. The baseline demographic and clinical data of the patients and controls are summarized in Table 1. eGFR was calculated with MDRD formula. The result showed that 4 of the patients were diagnosed with stage 1 CKD, 4 with stage 3, 4 with stage 4, and 2 with stage 5 CKD.

Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis (NTA) of purified urine showed the presence of exosomes and many microparticles. Exosomes were defined as small vesicles with a size of 30-120nm. The concentration of microparticles was greater than exosomes with some purified urines containing only microparticles and no exosomes. Although NTA was performed on all patient samples, only diagrams of 4 patients representative of each CKD stage were shown in the appendix (Diagrams 1-4). On average, patients in CKD stage 3 and 4 had more exosomes (~3 fold more) compared to CKD 1. However, the two patients in CKD 5 had no exosomes but lots of microparticles.

miRNA expression profiling

I investigated the profile of miRNAs in total urine and in the purified exosomes from the urine sample to discover if there were any similarities or differences. A total of 60 miRNA were detectable in our patient urine samples. 46 miRNA were in urinary exosomes and 29 were found in urine. Both populations had mostly different types of miRNA with only 15 miRNA shared between them. The miRNA profiled in our patient urine samples are listed in Table 2. A Venn diagram was drawn to show the relationship between the two populations (Diagram 5).

Taqman qRT-PCR

Out of the 60 miRNAs detected, miR-21 and miR-29c were chosen for further analysis by qRT-PCR in all recruited patients. Both were selected as there are previous studies on their role as biomarkers for chronic renal disease. We hoped that this would serve to validate the reproducibility of our results. My results show that miRNAs are expressed at a higher level in the purified exosomes than the urine sample. This is likely due to measuring miRNAs in purified exosomes obtained from the urine, rather than the urine as a whole. The changes were similar in the exosomes and urine but more dymanic changes were observed in the exosomes. The levels of miR-21 in urinary exosomes were upregulated in the urine and the exosomes as CKD stage increased. The purified exosomes demonstrated a more dymanic increase in the expression of miR-21. However, the levels for miR-29c in our patients vary only slightly across all CKD levels. This was contrary to the findings of a paper where they reported reduced levels of miR-29c in CKD patients. (Table 3)

3.7 Discussion (500 words max):

This pilot study demonstrated that the exosomes samples contained more miRNA than whole urine samples. This could be because the exosomes samples were purified and hence contain concentrated levels of miRNA compared to the more dilute whole urine samples. This raises the question if purified exosomes are the best method for measuring miRNA levels as it might result in an overestimation. However, current consensus is that urinary exosomes provide a full representation of the entire urinary system [97] because they are normally secreted by cells from all nephron segments

Another interesting discovery was that the exosomes sample extracted by Total Exosome Isolation (from urine) reagent (Life Technologies) contain not only exosomes, but microparticles as well. The concentration of microparticles was greater than exosomes in all patients. Furthermore, some samples contain only microparticles and no exosomes. The function of microparticles in urine is not well understood and it could be that some miRNA are packaged in them. Therefore, further research should be done on the role of urinary microparticles to determine if their concentration has any clinical significance.

I discovered that levels of miR-21 in purified exosomes and whole urine samples correlate with CKD stage. However, a larger sample size is needed for the study to be adequately powered before statistical analysis can be carried out. I was also not able to determine which factors predict miRNA-21 levels in patients undergoing kidney transplant biopsy compared to native kidney biopsy due to the small patient sample size. As this pilot included many causes of CKD, it could be concluded that miRNAs are a robust biomarker for renal damage.

Using the miRNeasy micro kit (Qiagen, Hilden, Germany), miRNAs can be extracted and measured in a short amount of time. Although more dymanic changes were observed in the exosomes, the changes in microRNA levels were mostly similar in both exosomes and urine. As exosomes do not need to be isolated before measure microRNA, levels of microRNA in a large cohort of patients can be measured easily. Therefore, it is possible for measurement of microRNA levels to be adapted in clinical practice.

Among the miR profiled in our urine sample, a few were of interest and may warrant further research. These microRNAs have been discussed in various literatures as potential biomarkers for chronic kidney disease and the presence of these microRNAs in our study further supports this hypothesis. MiR-16, miR-141, miR-192, miR-20a and miR-223 are examples of miR of interest as their levels were shown to increase in Wistar rats with cisplastin-induced kidney injury.

In two studies conducted in mice, i.e. after induction of acute and chronic kidney injury with renal ischemia-reperfusion and strep-tozotocin treatment, respectively, miR-10a and -30d were found significantly increased in urine (Wang et al., 2012b). Since they are generally highly expressed in kidneys, they were suggested as possible candidates for non-invasive and specific urinary markers of renal injury. In another study, miR-30d levels in urine was also found to increase after induction of kidney damage by cisplastin.

A similar study to ours was performed by Ramachandran et al. where they profiled the miRNAs in urine of patients with acute kidney injury (AKI) compared to healthy volunteers. The research led to the identification of 4 miRNAs capable of significantly differentiating patients with AKI from individuals without AKI. (Ramachandran et al., 2013). It is interesting to note that none of these miRNA was detected in our study.

4. Reflection by the student on the experience and value of the studentship (300 words max):

As a medical student, working in a research laboratory was a mystery to me. It was the place where blood samples were sent for analysis to investigate novel methods for diagnosis and understanding disease pathophysiology using tissue samples. This narrowed view of what a laboratory does was thankfully changed as I had the opportunity to experience life as a researcher through this summer studentship. It is not an overstatement to say that my eyes were opened to a whole new world and culture. Although it was a challenge in the beginning, I found that working in a lab became more enjoyable as the days went on. This was due to the help and support not only by Dr. Denby, but also by fellow researchers who were very approachable.

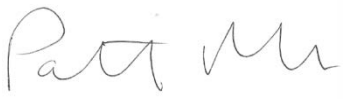
Fully aware that I had no experience in analysing blood samples, she taught me the various skills needed and gave me time to practise before we proceeded to actual patient samples. When my experiments failed, Dr Denby never put me down but encouraged me to do better for the next one. With each trial and error, I grew in confidence and was finally able to carry out experiments on my own. Dr Denby would explain the concepts and reasons behind each procedure so that I could understand instead of blindly following it. I am glad to have learnt many research techniques which are not taught in my course.

I have also learnt first-hand how temperamental science can be as experiments can sometimes fail for no reason at all. Although this was disheartening, it also served to accentuate the satisfaction felt when a good result is achieved. I thoroughly enjoyed working in a laboratory environment where everyone selflessly helps each other in the common pursuit of knowledge.

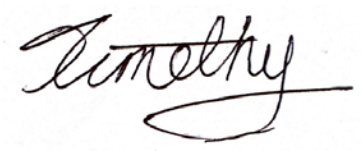
This summer studentship has indeed opened up the possibility of me pursuing an intercalated degree and maybe an academic foundation programme when I graduate as a doctor. I am grateful for the knowledge, skill and mentorship obtained through it

5. Dissemination: (note any presentations/publications submitted/planned from the work):

6. Signatures: Supervisor Date Student Date



15th August 2014



15th August 2014

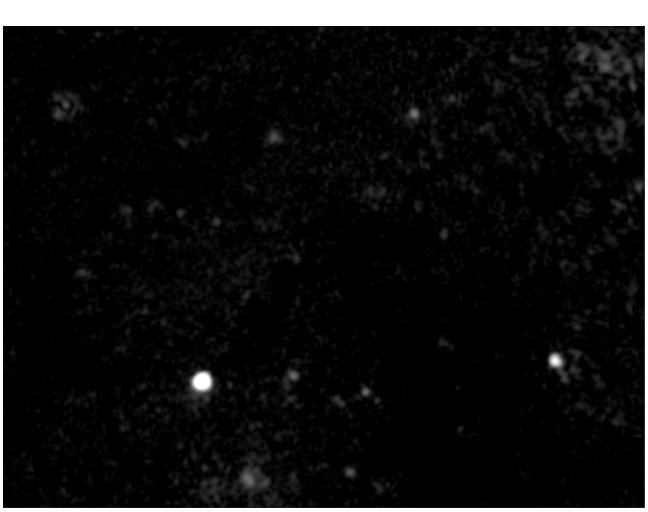
Appendix

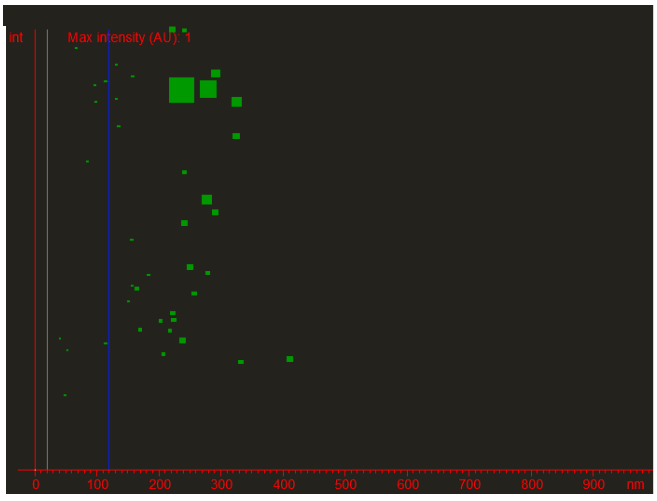
Table 1: Patient demographics

	CKD 1 (n=4)	CKD 3 (n= 4)	CKD 4 (n= 4)	CKD 5 (n= 2)
Age, yr	52.50 (19.33)	55.25 (11.00)	42.50 (11.39)	59.50 (6.36)
Sex, male/female	1/3	4/0	2/2	2/0
eGFR	93.23 (0.79)	38.00 (7.79)	20.75 (3.20)	9.50 (4.95)
Creatinine	67.00 (9.31)	168.75 (28.65)	269.00 (79.54)	630.50 (320.32)
PCR	501.75 (442.57)	614.75 (782.39)	298.25 (313.44)	341.00 (417.19)

Values are Mean (Standard deviation); eGFR, estimated glomerular filtration rate; PCR, protein-creatinine ratio

Diagram 1: CKD 1 Patient Purified Urine

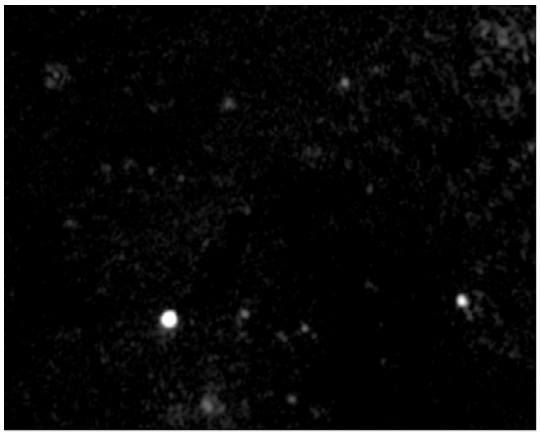


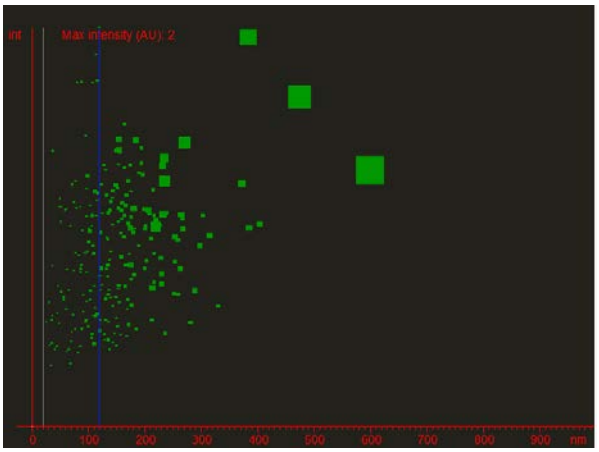


Total concentration: 5.89E8 particles / ml

Selected concentration: 0.28E8 particles / ml

Diagrams 2: CKD 3 Patient Purified Urine

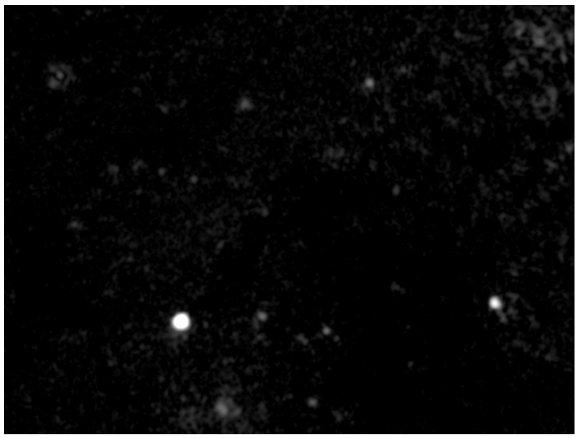


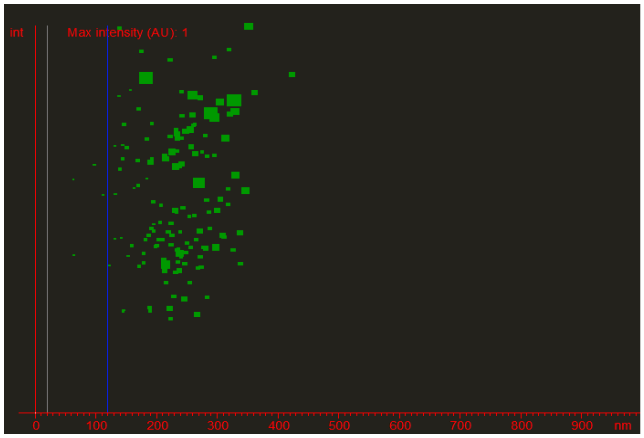


Total concentration: 11.19E8 particles / ml

Selected concentration: 1.95E8 particles / ml

Diagrams 3: CKD 4 Patient Purified Urine

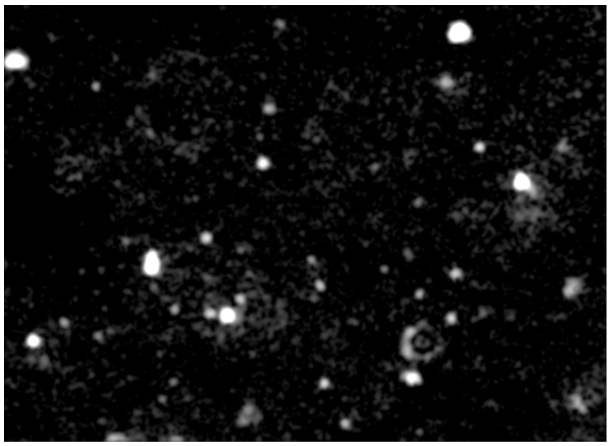


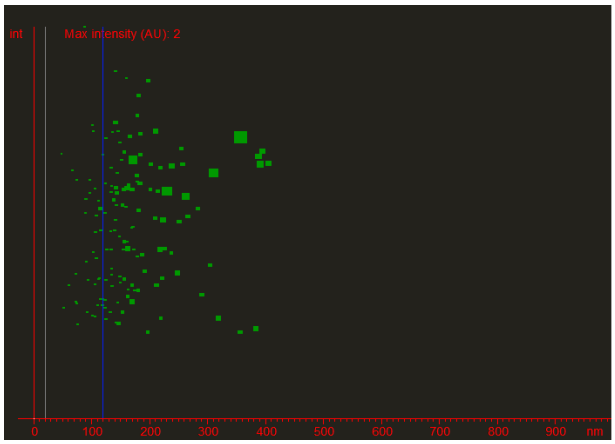


Total concentration: 6.48E8 particles / ml

Selected concentration: 0.06E8 particles / ml

Diagram 4: CKD 4 Patient Purified Urine





Total concentration: 12.78E8 particles / ml

Selected concentration: 1.38E8 particles / ml

Table 2: List of Types of microRNA in urine sediments, exosomes and those shared between both populations

Free microRNA in urine	microRNA in exosome	Common elements in "Urine miRs" and "Exosome miRs"
hsa-let-7c	hsa-let-7c	hsa-let-7c
hsa-miR-10a	hsa-miR-10a	hsa-miR-10a
hsa-miR-16	hsa-miR-16	hsa-miR-16
hsa-miR-19b	hsa-miR-21	hsa-miR-21
hsa-miR-21	hsa-miR-26a	hsa-miR-26a
hsa-miR-24	hsa-miR-26b	hsa-miR-92a
hsa-miR-26a	hsa-miR-34a	hsa-miR-106b
hsa-miR-92a	hsa-miR-92a	hsa-miR-192
hsa-miR-99b	hsa-miR-106b	hsa-miR-20a
hsa-miR-106b	hsa-miR-125b	hsa-miR-29c
hsa-miR-152	hsa-miR-130a	hsa-miR-106a
hsa-miR-192	hsa-miR-135a	hsa-miR-31
hsa-miR-194	hsa-miR-141	hsa-miR-191
		hsa-miR-30a-3p

hsa-miR-203 hsa-miR-375 hsa-miR-20a hsa-miR-29c hsa-miR-30b hsa-miR-29a hsa-miR-106a hsa-miR-886-5p hsa-miR-31 hsa-miR-223 hsa-miR-191 hsa-miR-17 hsa-miR-30a-3p hsa-miR-30a-5p HSA-MIR-1274B HSA-MIR-720	hsa-miR-148b hsa-miR-192 hsa-miR-205 hsa-miR-361 hsa-miR-374 hsa-miR-20a hsa-miR-29c hsa-miR-146b mmu-miR-451 hsa-miR-660 hsa-miR-532 hsa-miR-324-3p hsa-miR-106a hsa-miR-345 hsa-miR-886-3p hsa-miR-10b hsa-miR-126 hsa-miR-135b hsa-miR-31 hsa-let-7d hsa-miR-191 hsa-miR-556-3p hsa-miR-30a-3p hsa-miR-30d hsa-miR-34b hsa-miR-601 hsa-miR-629 hsa-miR-607 hsa-miR-505# hsa-miR-223# hsa-miR-378 hsa-miR-10b# HSA-MIR-720	HSA-MIR-720

Diagram 5: Venn Diagram of number of microRNA in urine sediments, exosomes and those shared between both populations

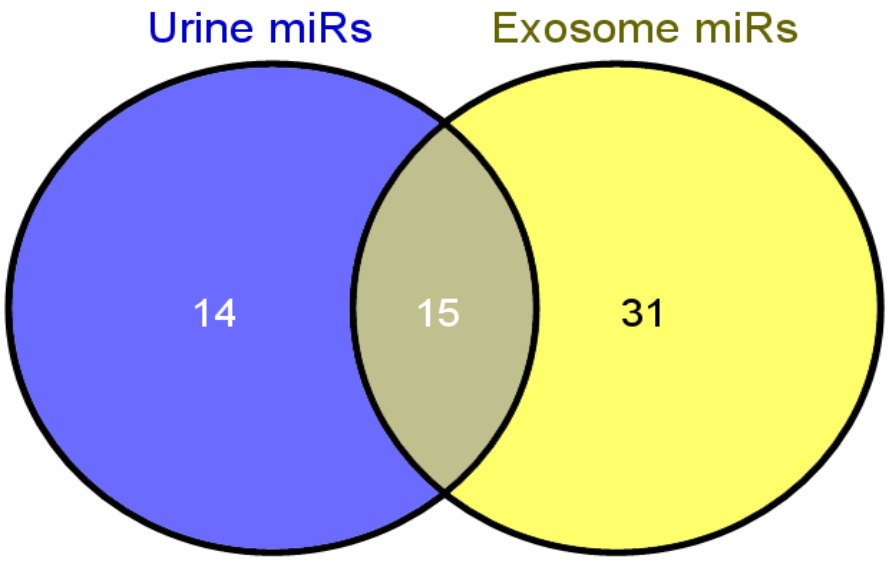


Table 3: Levels of miR-21 and miR-29c according to CKD stage

MicroRNA	Levels of miRNA according to CKD stage			
	CKD 1	CKD 3	CKD 4	CKD 5
MiR-21	28.08 (2.02)	26.74 (2.54)	26.98 (1.74)	25.34 (2.69)
MiR-29c	30.68 (2.53)	29.88 (2.48)	30.54 (1.85)	29.33 (2.44)