Université Fédérale
Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par :
Theofilos PAPADOPOULOS
le lundi 28 novembre 2016

Titre :

miRNAs in kidney disease

École doctorale et discipline ou spécialité :
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Dr Joost Peter Schanstra (Directeur de thèse)
Dr Julie Klein (Co-directrice de thèse - invité)

Université de Toulouse

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Délivré par *l'Université Toulouse III - Paul Sabatier*
Discipline ou spécialité : *Innovations pharmacologiques*

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This PhD thesis is dedicated to my parents Katerina and Nikos.

Abstract

MicroRNAs are now recognized as key players in the regulation of proteins and any abnormality in their function is a cause for pathway instability, leading to pathological conditions. Numerous reports from a variety of pathologies provide new data about microRNAs function, their targets and their potential as biomarkers and possible ways to control microRNAs' expression for potential therapeutic purpose.

A number of reports also connect microRNAs with pathological conditions in the kidney and point to the use of microRNAs as biomarkers for diagnosis and prognosis of kidney disease in blood, serum, tissue and urine samples. In this thesis, we researched:

1) A possible role of the microRNAs in the progression of adult chronic kidney disease (CKD), a disease representing a global burden with the tendency to rise worldwide. Progression of CKD is still very hard to detect non-invasively with the currently used clinical tools (eGFR and albuminuria). In our work we studied alterations of the level of the microRNAs in human urine samples of patients with fast or slow progression of CKD, in order to identify new potential biomarkers for non-invasive progression of CKD. Using Next Generation Sequencing, we analyzed urinary microRNA modifications in urine samples of 70 patients with established CKD and correlated their expression profiles to disease progression. This lead to the identification of 25 urinary microRNAs significantly associated to CKD progression (adjusted pvalue<0.05). Among those, four microRNAs (hsa-miR-34c-5p, hsa-miR-410-3p, hsa-miR-301b-3p, and hsa-miR-145-5p) were selected for validation in an independent cohort of 52 patients with CKD. Increased urinary abundance of hsa-miR-145-5p was confirmed to be associated to progression of CKD. *In vitro* exploration of the effects of hsa-miR-145-5p inhibition in human kidney cells showed that the microRNA seemed to be involved in necrotic processes. In conclusion we have identified hsa-miR-145-5p as potential urinary microRNA marker of CKD progression.

2) The identification of microRNAs associated to obstructive nephropathy, a frequently encountered disease in children that can lead, in severe cases, to end stage renal disease (ESRD). In this study we used a comprehensive system biology analysis in which we combined micro- and mRNA data from human and animal obstructive nephropathy to obtain information on possible mechanisms involved in this disease. In particular, we have studied in parallel the urinary miRNome of infants with ureteropelvic junction (UPJ) obstruction and the kidney tissue miRNome and transcriptome of the corresponding neonatal partial unilateral ureteral obstruction (UUO) mouse model. Several hundreds of microRNAs and mRNAs displayed changed

abundance during disease. Combination of microRNAs in both species and associated mRNAs let to the prioritization of 5 microRNAs and 35 mRNAs associated to disease. *In vitro* and *in vivo* validation identified consistent dysregulation of let-7a-5p and miR-29-3p and new potential targets, E3 ubiquitin-protein ligase (DTX4) and neuron navigator 1 (NAV1). Our study is the first to correlate a mouse model of neonatal partial UUO with human UPJ obstruction in a comprehensive systems biology analysis. Our data revealed let-7a and miR-29b as molecules potentially involved in the development of fibrosis in UPJ obstruction via the control of DTX4 in both man and mice that would not be identified otherwise.

Résumé

A l'heure actuelle, les microARNs sont reconnus comme des régulateurs essentiels de l'expression des protéines. Des anomalies dans leur fonction sont associées au développement de nombreuses pathologies. Ainsi, de nombreuses études s'intéressent au potentiel des microARNs en tant que biomarqueurs ou cibles thérapeutiques dans une grande variété de pathologies.

De plus, un certain nombre de recherches font le lien entre microARNs et pathologies rénales et soulignent l'intérêt des microARNs en tant que biomarqueurs pour améliorer le diagnostic et le pronostic des maladies rénales en utilisant des échantillons sanguins, sériques, de tissus et d'urine.

Dans le cadre de cette thèse, nous avons étudié :

1) L'association des microARNs urinaires avec l'évolution de la maladie rénale chronique (MRC) chez l'adulte. La prévalence de la MRC est actuellement estimée à 5-10% de la population et est en constante augmentation. La détection précoce et l'identification de patients ayant une MRC progressant rapidement vers l'insuffisance rénale sont la clé pour une meilleure prise en charge de ces patients. Actuellement les outils non-invasifs comme l'albuminurie ou l'estimation du débit de filtration glomérulaire manquent de précision. Dans notre travail, nous avons tenté d'identifier les modifications urinaires des microRNAs afin d'identifier de nouveaux biomarqueurs non-invasifs associés à la progression de la MRC.

Nous avons analysé les modifications des microARNs urinaires par séquençage à haut débit dans des échantillons d'urine de 70 patients atteints de MRC et corrélé leurs profils d'expression à la progression de la maladie. Cela a amené à l'identification de 25 microARNs urinaires (pvalue ajustée <0.05) potentiellement associés à la progression de la MRC. Parmi ceux-là, quatre microARNs (hsa-miR-34c-5p, hsa-miR-410-3p, hsa-miR-301b-3p, and hsa-miR-145-5p) ont été sélectionnés pour être validés dans une cohorte indépendante de 52 patients atteints de MRC. L'augmentation de l'abondance urinaire de hsa-miR-145-5p a été confirmée comme étant associée à la progression de la MRC. Des analyses *in vitro* de l'effet de l'inhibition de hsa-miR-145-5p dans les cellules rénales ont mis en évidence que ce microARN semblait être impliqué dans le processus de nécrose. En conclusion, cette étude nous a permis d'identifier hsa-miR-145-5p comme marqueur potentiel de la progression de la MRC.

2) La présence de microARNs urinaires associés à la néphropathie obstructive, une maladie fréquemment rencontrée chez les enfants qui peut conduire, dans les cas graves, à l'insuffisance

rénale précoce. Dans cette étude, nous avons utilisé la biologie des systèmes et avons combiné des données microARN et ARNm de néphropathie obstructive humaine et animale pour obtenir des informations sur les mécanismes possibles impliqués dans cette maladie. En particulier, nous avons étudié simultanément le miRNome urinaire de nourrissons présentant une obstruction de la jonction pyélo-urétérale et le miRNome et le transcriptome tissulaire rénal chez la souris dans le modèle animal d'obstruction urétérale unilatéral (OUU) partiel et néonatal. Plusieurs centaines de microARNs et d'ARNms étant modifiés, la combinaison des microARNs des deux espèces avec les ARNms cibles associés a permis de sélectionner les 5 microARNs et 35 ARNms les plus fortement associés à la néphropathie obstructive. Une validation *in vitro* et *in vivo* a mis en avant que let-7a-5p et miR-29-3p ainsi que deux nouvelles cibles potentielles, l'*E3 ubiquitin-protein ligase* (DTX4) et *neuron navigator 1* (NAV1) étaient dérégulées au cours de cette pathologie. Cette étude est la première à corréler le modèle animal d'OUU partiel et néonatal avec l'obstruction pyélo-urétérale chez l'Homme dans une analyse intégrée de biologie des systèmes. Nos résultats ont révélé let-7a et miR-29b en tant que molécules potentiellement impliquées dans le développement de la fibrose dans la néphropathie obstructive via le contrôle de DTX4 chez l'homme et la souris, ce qui n'aurait pas été identifiable autrement.

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Abbreviations

UTR: untranslated region

DGCR8: DiGeorge Syndrome Critical Region 8

AGO2: Argonaute 2

RISC: RNA-induced silencing complex

NGS: Next Generation Sequencing

TLDA: TaqMan Low-Density Arrays

LNA: locked nucleic acid

CKD: chronic kidney disease

ESRD: end-stage renal disease

eGFR: estimated glomerular filtration rate

EMT: epithelial-to- mesenchymal transition

TGF: Transforming growth factor

CAKUT: congenital anomalies of the kidney and the urinary tract

CON: congenital obstructive nephropathy

UPJ: ureteropelvic junction

UUO: unilateral ureteral obstruction

IPA: Ingenuity Pathway Analysis

DXT4: E3 ubiquitin-protein ligase (Deltex 4)

LMOD1: leiomodin-1

ADAM19: ADAM Metallopeptidase With Thrombospondin Type 1 Motif 19

NAV1: neuron navigator 1

HK2: Human Kidney cells

IL-6: Interleukin-6

1. miRNAs

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MiRNAs are short (18-23 nucleotides in length), non-coding, endogenous, single-stranded RNA molecules involved in post-transcriptional regulation of gene expression. Discovered 2 decades ago, these small RNA molecules are one of the "hottest" trends in biological research.

1.1 A little bit of history

The discovery of the first miRNA took place in 1993. In that year, the two teams of Ambros and Ruvkun independently published complementary studies on a gene, called lin-4 in Caenorhabditis elegans **(Lee et al., 1993, Wightman et al., 1993)**. Both teams identified that the lin-4 gene produces a small transcript that does not encode a protein but has complementary sequence with the 3'-UTR region of lin-14 gene and negatively regulates the level of the LIN-14 protein involved in the development of *C.* Elegans **(Lee et al., 1993, Wightman et al., 1993)**. These two groups had identified the first miRNA! Seven years had to pass until the identification of a second miRNA, let-7 **(Reinhart et al., 2000)**.

In 2006, the Nobel Prize in Physiology or Medicine was jointly given to Fire and Mello for their research in 1998 on the discovery of the silencing mechanism of mRNA expression by small interfering RNA molecules, now known as the action mechanism of miRNAs. Since 2001, miRNAs or miRNAs have been shown to be involved in a variety of physiological functions and diseases and forced the scientific interest and research to evolve accordingly towards detecting and identifying these small molecules **(Almeida et al., 2011)**. The continuously increasing number of reports on miRNAs, starting from some hundreds per year until 2007 and reaching a total of approximately 9000/year in 2015 (PubMed search for "miRNAs" and "miRNA"), demonstrates the growing interest for these small molecules in physiology and disease.

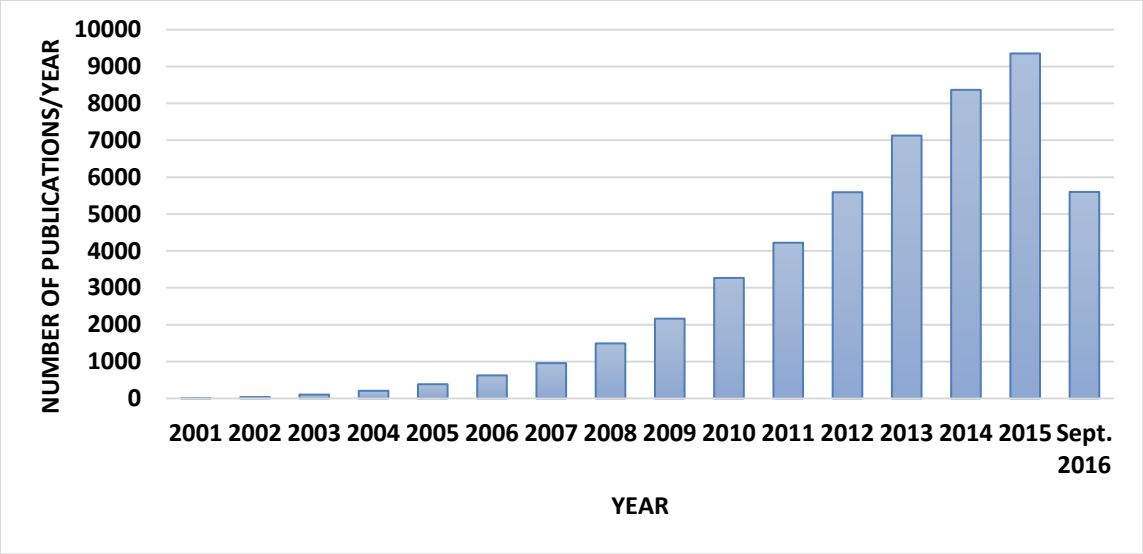

Figure 1: The number of total publications/year on miRNAs has been continuously increasing over the years.

This increase is also the result of the more extensive use of Next Generation Sequencing (NGS) technologies in combination with simplified extraction, quantification and expression analysis methods of miRNAs (see chapter "Research methods" on page 18). Improved bioinformatics tools have also contributed to the number of newly identified miRNAs **(Friedlander et al., 2014)**. Today, the number of miRNA entries in miRBase (the first and largest database of miRNAs) in the last update of June 2014 (version 21.0) reached 28645 miRNA entries when considering all species, and 2588 entries for human miRNAs only **(Kozomara and Griffiths-Jones, 2014)** .

1.2 Biogenesis and function

The biogenesis of a miRNA is initiated with the generation of a so-called primary miRNA (pri-miRNA) in the nucleus, which is reformed to the precursor miRNA (pre-miRNA) before it leaves the nucleus **(Bartel, 2004)**. Then, after some other maturation steps the pre-miRNA is being transformed to the mature miRNA ready to perform its biological function: bind to the mRNA - target and suppress its expression **(Hammond, 2015)**. The whole process of miRNA biogenesis and the function mechanism has been extensively studied and are summarized in Figure 2 **(Hammond, 2015, Winter et al., 2009, Meister, 2013, Macfarlane and Murphy, 2010, Lewkowicz et al., 2015, Ha and Kim, 2014, Eulalio et al., 2009)**.

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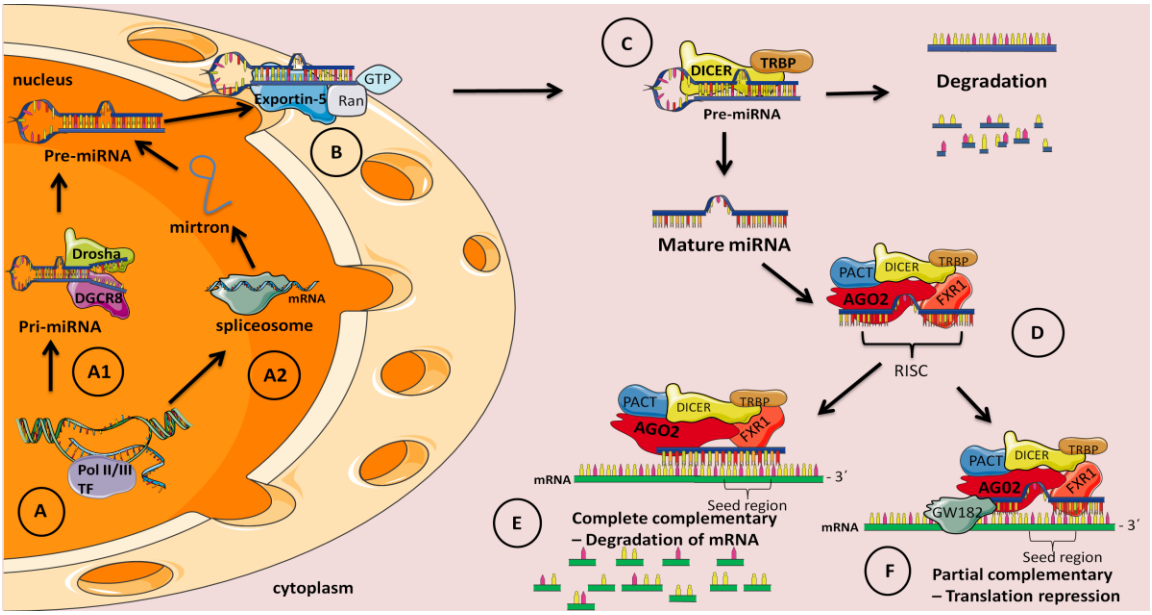


Figure 2: miRNA biogenesis and function. A1) Canonical pathway: miRNA is transcribed in a primary form (pri-miRNA with a hairpin loop) with the help of Polymerase II or III and various transcription factors and then spliced by the Drosha/DGCR8 complex to form the precursor miRNA (pre-miRNA). **A2) Non canonical pathway:** a "mirtron" is generated from mRNA after splicing in a complex called spliceosome. Next, the mirtron reforms to the shape of a pre-miRNA. **B)** The pre-miRNA is transferred from the nucleus to the cytoplasm with the help of Exportin-5 in a Ran-GTP dependent manner. **C)** In the cytoplasm the protein DICER together with TRBP cleaves the pre-miRNA hairpin loop and releases the two strands. One strand will serve as the mature miRNA (18 - 22 nt length), while the other, in most cases, will be degraded. **D)** The mature miRNA then binds to a protein complex including AGO2, forming the so called RISC complex. AGO2 then leads the miRNA to the mRNA target: **E)** if the miRNA sequence is completely complementary to the 3'-UTR of the mRNA, it binds and AGO2 cleaves the mRNA, completely stopping the translation of the protein. **F)** if the miRNA sequence is partially complementary with the mRNA's 3'-UTR, GW182 is recruited in the RISC complex to aid with the connection and inhibit translation.
Abbreviations: DGCR8 (DiGeorge Syndrome Critical Region 8), TRBP (HIV-1 transactivating response (TAR) RNA-binding protein), PACT (Protein Kinase R-activating protein), FXR1 (fragile X mental retardation-related protein 1), AGO2 (Argonaute 2), RISC (RNA-induced silencing complex). The picture was designed using Servier Medical Art http://smart.servier.fr/servier-medical-art

1.3 miRNAs in physiology

MiRNAs play a significant role during organ development. It has been reported by Tang *et al.* that 54 miRNAs are expressed in higher levels in human foetal organs compared to adult matched organs **(Tang et al., 2011)**. Furthermore, the importance of the miRNAs in keeping the homeostasis on the human body is reflected by the fact that any dysregulation in their expression leads to pathological conditions. The most characteristic example of that derives from mouse models deficient for Dicer and Drosha, two proteins with key roles in miRNA biogenesis. In particular, it has been shown that in knockout mice for these molecules leads to undeveloped organs and/or lethality **(Bernstein et al., 2003, Wang et al., 2007, Park et al., 2010).**

More experiments in animal models have supported the concept that miRNAs are involved in numerous cellular events during animal development by controlling key processes including cell fate determination, proliferation and cell death **(Stefani and Slack, 2008).** For example, let-7 (the second ever discovered miRNA) is a well-studied miRNA that has been found to control cell proliferation via regulation of apoptosis and has been linked to different forms of cancer due to its dysregulation **(Sun et al., 2016)**. MiR-203 is responsible for skin differentiation by repressing p63 in stratified epithelial cells and miR-196 is involved in the development of hind limb **(Hornstein et al., 2005, Chen et al., 2016)**. Other examples of miRNAs involved in physiological processes include miR-124, a miRNA expressed specifically and abundantly in the mouse brain and helping to maintain the neuronal cellular identity **(Sempere et al., 2004)**, miR-134, an important actor for synaptic development and plasticity **(Schratt et al., 2006),** miR-1, for its role in controlling the development of skeletal and heart muscle **(Zhao et al., 2005)** and miR-181 and miR-155 in lymphocyte development **(Li et al., 2007, Metzler et al., 2004).** Finally, miRNAs are also involved in immune response, insulin secretion, neurotransmitter synthesis and viral replication **(Wijesekara et al., 2012, Greco and Rameshwar, 2007, Gantier et al., 2007, Kim et al., 2016)**.

1.4 Annotation

The nomenclature of miRNAs is complex. In 2003 Ambros et al. proposed guidelines for newly identified miRNA annotation **(Ambros et al., 2003)**. These rules were applied later to miRBase and are followed until today for every new miRNA identified. The rules are summarized in Figure 3 **(Griffiths-Jones, 2004)**.

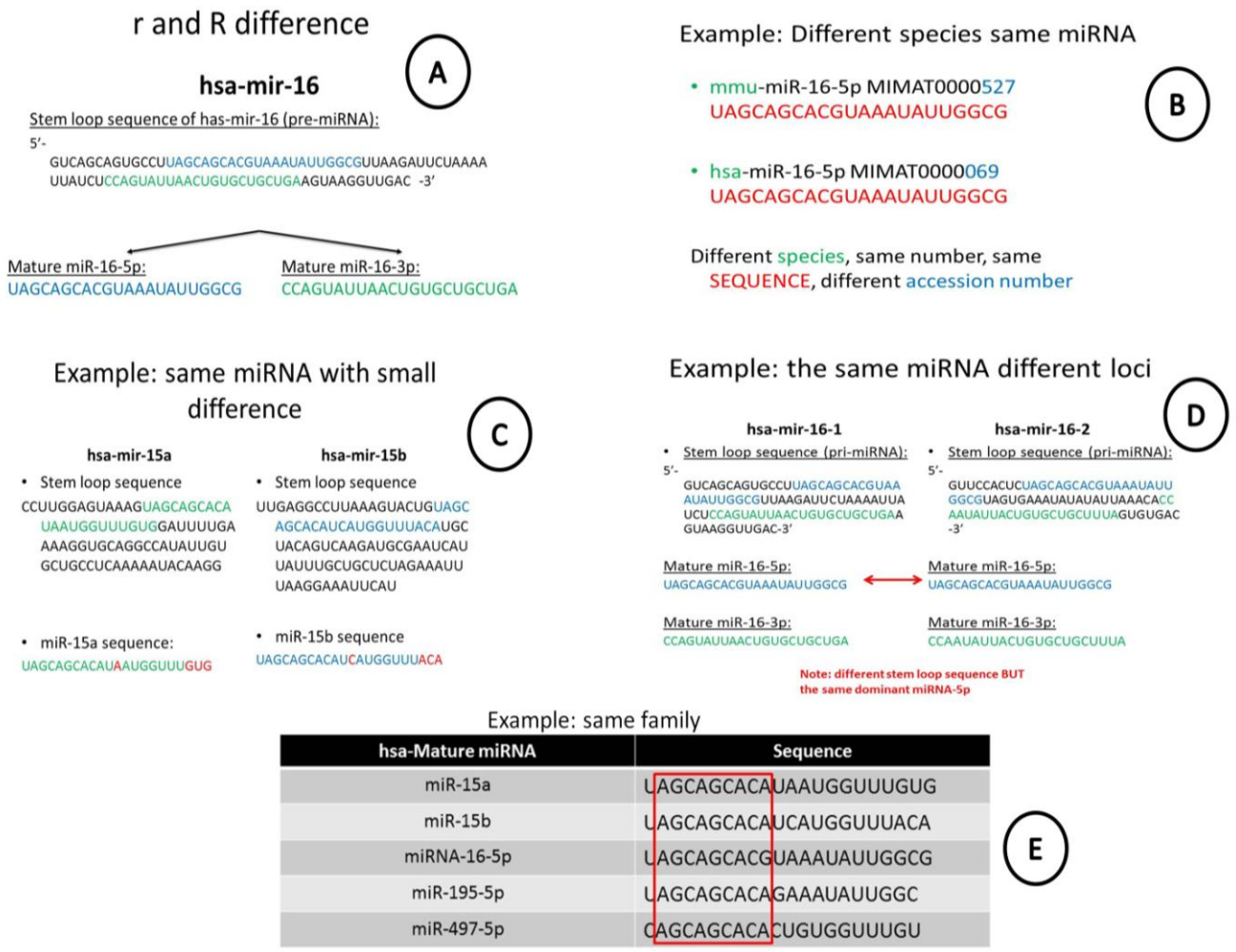


Figure 3: Examples of the miRNA annotation. A) mir and miR: a small (r) represents the pre-miRNA while the (R) represents the mature miRNA, e.g. mir-16 is the precursor miRNA-16 and miR-15 the mature miRNA. Recent studies however suggested that not in all cases the mature miRNA is released from the same arm **(Guo et al., 2015)** and as a result the suffix -5p or -3p is added to define from which arm of the pre-miRNA the mature miRNA is originating. B) The three letters in front of the miR represents the species in which a particular miRNA is observed; e.g. hsa (homo-sapiens), mmu (mus musculus). The numbers to register the miRNAs are ascending. The same miRNA in different species receives the same number to preserve homology among the database, e.g. hsa-miR-16-5p (human) is the ortholog of mmu-miR-16-5p (mouse). Different numbers are given when a miRNA has significant sequence differences. The accession number of each miRNA is the only unique identifier. Lin-4 and let-7 (lethal-7) are an exception for historical reasons. C) Sequences with one or 2 different nucleotides are assigned with the same number but an additional letter to distinguish; e.g. hsa-miR-15a and hsa-miR-15b. D) If the same miRNA is found in different loci of a chromosome, the difference is indicated from the pre-miRNA level, a number is added to distinguish those; e.g. hsa-mir-16-1 and hsa-mir-16-2. E) If the miRNAs share the same sequence over a stretch of 2-8 nucleic acids, they are derived from the same precursor and belong to the same cluster generating a miRNA family; e.g. the mir-15 family consists of miR-15a and miR-15b sequences, as well as miR-16-1, miR-16-2, miR-195 and miR-497.

1.5 Location

1.5.1 Tissue distribution

Numerous reports, using a variety of molecular biology methods and *in-silico* analysis, provide information about the expression levels of miRNAs in different tissues **(Liang et al., 2007, Tang et al., 2011, Sood et al., 2006)**. This analysis led to the suggestion that miRNAs distribute unequally in tissue and that some miRNAs can be listed as "tissue specific" and can be used as identifiers for these tissues. For example, miR-122a is specific for the liver, miR-1 and miR-133a for the heart and skeletal muscle, miR-9 for the brain **(Sood et al., 2006)**, and miR-192 and miR-194 for gastrointestinal organs and kidney **(Liang et al., 2007).**

1.5.2 Bodyfluids

In 2009 Hanson et al. evaluated for the first time miRNA expression in five dried body fluids including blood, saliva, semen, vaginal secretions and menstrual blood **(Hanson et al., 2009)**. They, in addition with others, were able to identify a number of miRNAs, which could be used to distinguish between different fluid samples. These miRNAs were **(Zubakov et al., 2010, Nakai et al., 2010, Isakova et al., 2011, Shinkai et al., 2008)**:

- miR-16, miR-20a, miR-126, miR-451 and miR-486 for blood
- miR-10b, miR-135a, miR-135b, miR-888, miR-891a and miR-943 for semen
- miR-138-2, miR-200c, miR-203, miR-205, miR-583 and miR-658 for saliva
- miR-124a, miR-372, miR-617 and miR819a for vaginal secretions
- miR-185, miR-214 and miR-412 (in combination with miR-451) for menstrual blood.

Further studies by Weber et al. showed that miRNAs can be found in at least twelve different body fluids including milk, colostrum, saliva, seminal fluid, tears, urine, amniotic fluid, bronchial lavage, cerebrospinal fluid, plasma, pleural fluid, and peritoneal fluid **(Weber et al., 2010)**.

But how do these miRNAs end up in body fluids? MiRNAs are secreted from the cells in which they are produced and packed in microparticles such as microvesicles, exosomes or apoptotic bodies and are also attached to RNA-binding proteins or lipoprotein complexes explaining their presence in body fluids (Figure 4) **(Beltrami et al., 2012).** Of note, except of being the means of transport, miRNA carriers and binding proteins, also act as "bodyguards" of miRNAs by protecting them from RNAse activity.

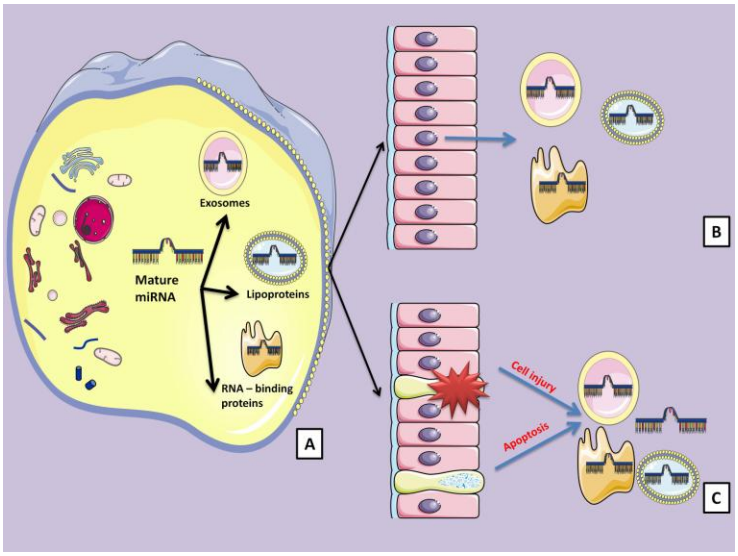


Figure 4: miRNA transporting molecules. A) During biogenesis or even after fulfilling their biological function, miRNAs can be incorporated into exosomes or bound to lipoproteins (e.g. HDL) or RNA-binding proteins (e.g. AGO2). Release of the encapsulated or bound miRNAs can occur via B) exocytosis or C) during cell lysis and apoptosis. Encapsulation or binding to these proteins most likely protects the miRNAs from degradation in the hostile biofluid environment.

Exosomes are the best-studied miRNA-vehicles. These vacuoles transfer cellular components between cells and promote cell-to-cell communication and interactions **(Camussi et al., 2010, Ramachandran and Palanisamy, 2012)**. Studies have shown the existence of exosomal transfer of functional mRNAs and miRNAs from one cell to another. One of the strongest arguments for the existence of this phenomenon is the study of Valadi et al. in which human mast cells were incubated with exosomes secreted by mouse mast cells for 24h. After the incubation, 92 mouse proteins were detected in the human cells showing that the exosomes carried mouse "functional" mRNAs into the human cells leading to the production of mouse proteins in the human cells **(Valadi et al., 2007)**. Studies reported that exosomal miRNAs are intact and protected from degradation rather than cell-free miRNAs which are more likely to be degraded by RNAses **(Cheng et al., 2014b) (Cheng et al., 2014a)**.

Moreover, RNA-binding protein AGO2 (Figure 4) was shown to be involved in miRNA transport in the circulation, which potentially leads to the transport of a functional miRNA-induced silencing complex **(Arroyo et al., 2011)**. Another RNA-binding protein with a similar action as AGO2 is nucleophosmin 1, which was found to carry miRNA in serum in humans and is possibly involved in cell-to-cell communication **(Wang et al., 2010b)**. HDL lipoproteins, surprisingly, were found to have a role in the transport of miRNAs in a similar way as RNA-binding proteins **(Vickers et al., 2011)**. Finally, circulating apoptotic cells seem to contribute to miRNAs transport as well. MiRNAs are released in the circulation after apoptosis bound to the previously mentioned RNA-binding proteins and can be absorbed by other cells and perform their function **(Turchinovich et al., 2011, Mlcochova et al., 2014)**.

1.5.3 miRNAs in urine

Urinary miRNAs are attached to RNA-binding proteins or contained in exosomes **(Lorenzen and Thum, 2012, Cheng et al., 2014b)**. One point that needs to be highlighted is that miRNAs in urine are less abundant than in plasma or serum and this is most likely due to higher RNase activity in urine **(Cheng et al., 2014b)**. The origin of urinary miRNAs is still not fully elucidated. Urinary miRNAs are likely to be shedded from cells all along the urinary pathway **(Fang et al., 2013a, Mlcochova et al., 2014)**, but could also potentially be filtered from the plasma, making the urinary pool of miRNAs potentially suitable for detection and monitoring of both renal and non-renal diseases. Although the exosome size (30 – 100 nm) is at the limit value for glomerular filtration (fenestrations' diameter is 60 - 80nm **(Satchell, 2013, Haraldsson et al., 2008)**), a recent study has shown that exosomes can cross other physiological barriers, such as the brain blood barrier despite their large size **(Alvarez-Erviti et al., 2011)**.

The role of urinary miRNAs is still unknown. Either urinary miRNAs can be considered as waste or/and miRNAs could use urine as a vehicle to move through the urinary tract and function in areas downstream from their site of production. Further research to answer these questions is needed.

1.6 Stability

Due to their small size and the protective mechanisms described above, miRNAs are quite stable **(Koberle et al., 2013, Gupta et al., 2010)**.

For research purposes, it is possible to extract miRNAs from biological samples stored at least up to one year under laboratory conditions (relatively constant humidity and ambient temperature, no UV exposure and dust free). MiRNAs extracted from such samples show no signs of degradation **(Zubakov et al., 2010)**. Plasma and serum miRNAs are stable for 24h at room temperature and resist to 8 cycles of freezing/thawing **(Mitchell et al., 2008)**. Further stability studies showed that plasma miRNAs extracted from exosomes are more stable compared to free plasma miRNAs when storing the samples at 4^{o}C, -20^{o}C and -80^{o}C for 2 weeks, 2 months, 3 years and 5 years **(Ge et al., 2014)** (Figure 5).

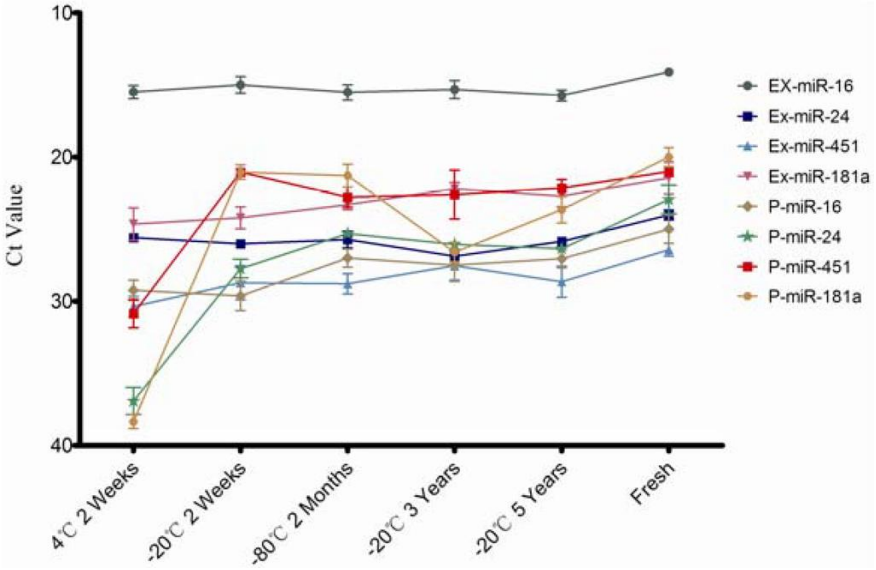


Figure 5: Stability of plasma exosomal and free miRNAs. Four abundant plasma miRNAs extracted from plasma exosomes (EX) and free plasma miRNAs (P). Exosomal plasma miRNAs are more stable compared to free plasma miRNAs **(Ge et al., 2014)**.

Urinary exosomes can also preserve miRNAs under different storage conditions as shown by *Yun et al*. In their study miR-200c extracted from urinary exosomes showed significant stability under different storage conditions and after multiple freeze-thawn circles **(Yun et al., 2012)** (Figure 6).

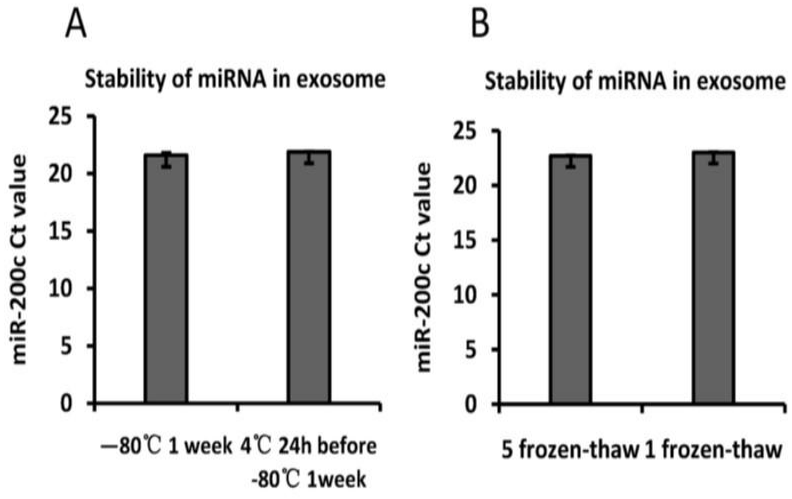


Figure 6: Stability of urinary exosomal miRNA (hsa-miR-200c). A) Analysis of miR-200c quantities in exosomes isolated from the same volume of pooled urine was 1) directly stored at -80℃ for 1 week or 2) stored at 4℃ for 24h, followed by storage at -80℃ for 1 week. B) Quantity of miR-200c after 5 and 1 Freeze-thaw cycles. **(Yun et al., 2012)**

Similar observations hold for formalin fixed paraffin embedded (FFPE) tissue samples. MiRNAs were found to be more stable compared to mRNA housekeeping genes under different tissue sample storage conditions and treatment, suggesting that miRNAs are more suitable than mRNAs as potential biomarkers **(Peiro-Chova et al., 2013)** (Figure 7).

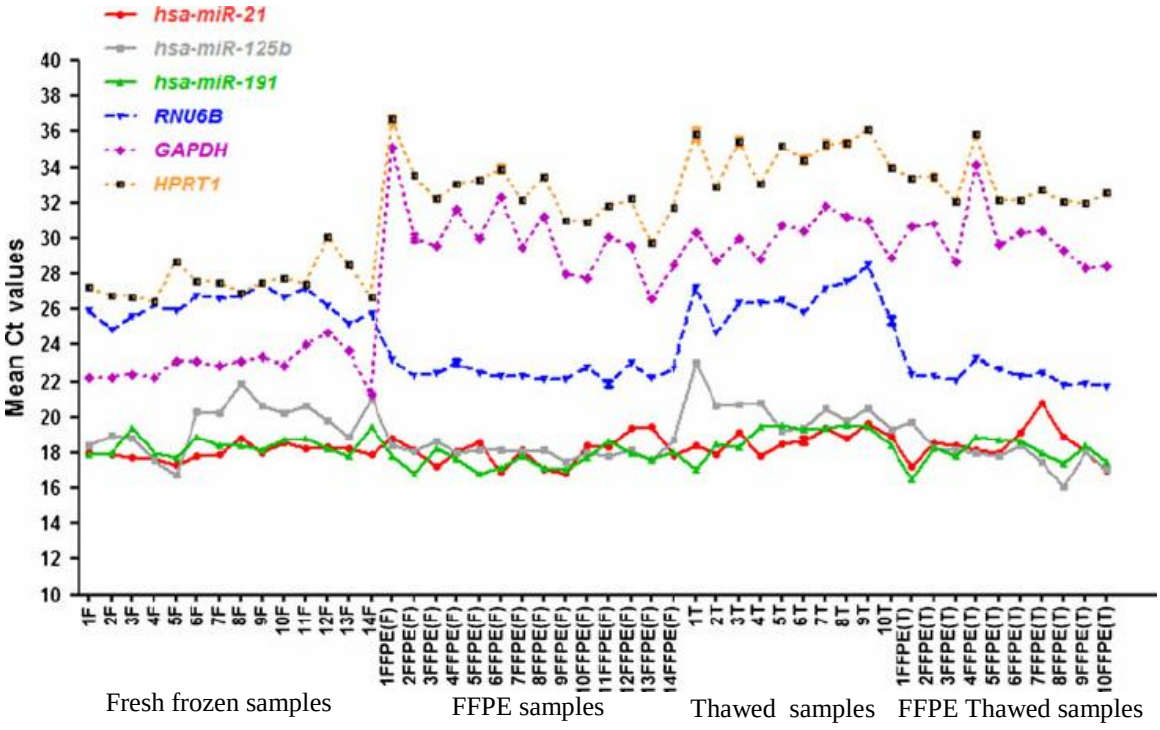


Figure 7: Diagram showing the stability of 3 miRNAs (hsa-miR-21, hsa-miR-125b and hsa-miR-191) and two commonly used mRNA housekeeping genes (GAPDH and HPRT1) in breast tissue samples under different conditions (fresh frozen, FFPE, thawed and FFPE thawed samples). Samples were paired. miRNAs of the same sample under these different conditions displayed a much more stable expression pattern than the housekeeping mRNA genes, GAPDH and HPRT1. Interestingly RNU6B, RNA often used for normalization of miRNAs, also displayed high variability compared to the miRNAs **(Peiro-Chova et al., 2013).**

The stability of free (non exosome encapsulated) urinary miRNAs has also been studied. One of the first studies that included the investigation of the stability of free miRNAs in urine was from Yun et al. They tested whether free miRNAs in urine can be used as prognostic and/or diagnostic biomarkers for bladder cancer. They showed that after 7 freeze-thaw cycles or after

storing urine at room temperature for 3 days, hsa-miR-145 and hsa-miR-200a showed only minimal signs of degradation (Figure 8) **(Yun et al., 2012)**.

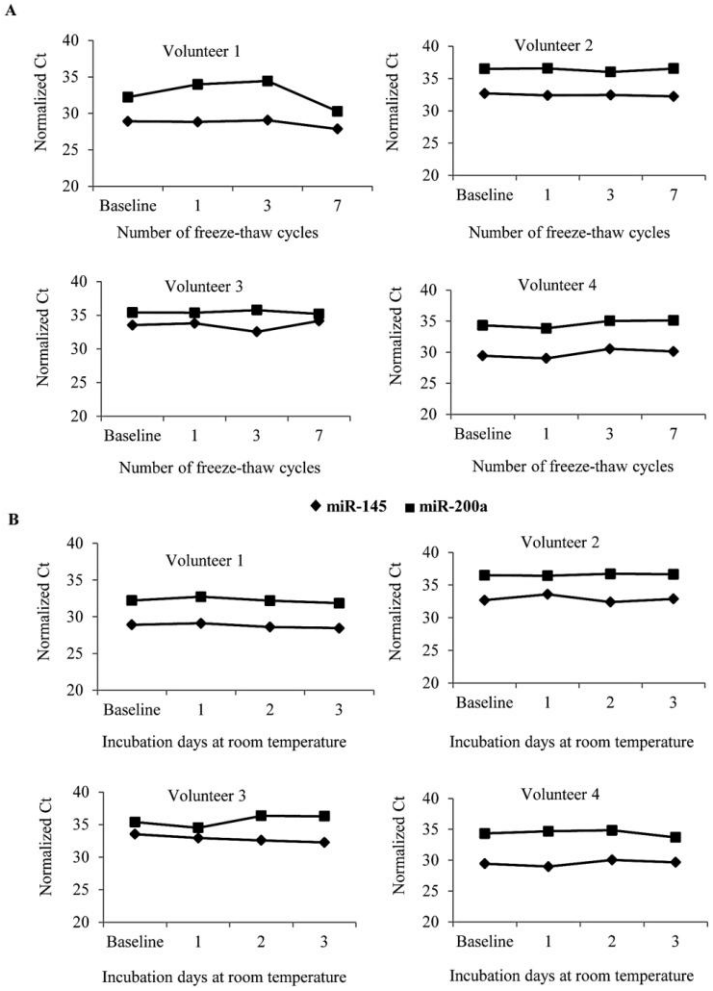


Figure 8: Free urinary hsa-miR-145 and hsa-miR-200a showing significant stability after A) 7 freeze and thaw cycles and B) over a period of 3 days in room temperature. (**Yun et al., 2012**)

Other studies demonstrated successfully the stability of urinary miRNAs after up to 10 freeze/thaw cycles, different storage temperatures between 4°C and -80°C for short and long periods of time (between 5 days to 2 years) **(Mall et al., 2013, Lv et al., 2013a)**.

This apparent stability of urinary miRNAs opens the window for multiple applications of miRNAs in research and in clinical practice.

Overall, the stability of miRNAs and their presence in nearly all tissues and body-fluids has placed miRNAs in the center of attention. Circulating miRNAs have become the study material of choice as possible biomarkers of disease.

1.7 Research methods

As discussed above high quality miRNAs can be extracted from a wide range of cells and tissue samples, such as cell lines and fresh or formalin-fixed paraffin-embedded (FFPE) tissue samples as well as any kind of body fluid (plasma, serum, urine etc.). Even if it was estimated that the miRNA fraction is about ~0.01% of the total RNA mass, the technological progress, together with the aforementioned stability of the miRNAs, yields in general enough miRNA material and of high quality for downstream studies (RT-qPCR, microarrays, next generation sequencing (NGS) etc.) **(Dong et al., 2013)**.

1.7.1 miRNA detection techniques

The techniques that are being used in miRNA expression analysis are similar to the techniques used in gene expression analysis with some modifications to adjust to the size and quantity of the miRNAs. Table 1 summarizes the features of the most important and popular detection techniques which will be briefly discussed below.

Northern Blot analysis was the first technique used to study the miRNAs, but the gold standard technology now in miRNA research is RT-qPCR. On the other hand, microarrays and Next Generation Sequencing (NGS) significantly boosted miRNA research, making it possible to detect and analyze multiple miRNAs in one sample simultaneously. New technologies have also been developed for miRNA detection that can potentially complement the "traditional" techniques. Such techniques are called biosensor techniques (BT). The difference between the use of BTs and the aforementioned technologies is the rapid and highly sensitive results from complex samples such as blood and urine, through the use of selective molecular probes, avoiding any need for polymerase-based amplification steps **(Johnson and Mutharasan, 2014)**.

All technologies have their advantages and disadvantages and a user must take under consideration all parameters before choosing the most suitable one. Sensitivity, cost, time to results, data processing are some of the most important parameters to consider. Even though NGS is winning the race of the most robust method for detection and discovery of new miRNAs, validation of findings with other technologies is currently mandatory.

Table 1: miRNA detection methods.

Method	Principle	Advantage	Disadvantage	Variants	References
Northern blot	Visualisation of the miRNA by electrophoresis with ^{32}P-labeled DNA probes	-Quantitative detection of mature miRNA, primary miRNA and precursor miRNA and miRNA complexes with Drosha, DICER and RISC	-Low sensitivity, -Low throughput, -Need of high RNA input	-(DIG)-labeled oligonucleotide probes with LNA, -EDC-mediated cross-linking, -LED, -DSLE	**(Lee et al., 1993, Ebhardt et al., 2010, Starega-Roslan et al., 2011, Koscianska et al., 2011, Johnson and Mutharasan, 2014, Pall et al., 2007, Kim et al., 2010)**
RT-qPCR	The extracted miRNAs are converted to the complementary DNAs generating cDNA followed by PCR amplification with specific primers to target the miRNA of interest	-Sensitivity, -Specificity -Reproducibility, -Low cost, -Simplicity, -Fast	-Lack of standardized normalization molecules, -Depends on purity and quality of RNA input, -Demanding design of the primers to deal with the small miRNA size	-miRNA specific or universal primers, -TaqMan probe or Sybr Green	**(Mirco et al., 2013, Dong et al., 2013, Hunt et al., 2015, Baker, 2010)**
Microarrays	Hybridization of the target miRNAs to the complementary fixed probes and detection via fluorescence	-Parallel analysis of hundred miRNAs in a single sample, -Easy, straightforward and automated analysis, -Short turn-around time, -Suitable for comparison between two conditions	-Prefixed probes - Not possible to detect new molecules, -Hybridization bias, -Semi-quantitative method, -Limited dynamic range	-´5 hairpin, -SHUT, -Label-free PAZ-dsRBD method, -LASH	**(Dong et al., 2013, Hunt et al., 2015, Pritchard et al., 2012, Chugh and Dittmer, 2012)**

Method	Principle	Advantage	Disadvantage	Variants	References
Next generation sequencing (NGS)	Massively parallel sequencing of millions of fragments of DNA from a single sample	-Suitable for detection of new molecules and miRNA heterogeneity -Increased dynamic range and sensitivity	-Size of data files, -Complex data analysis, -Long time, -Costly	-Semiconductor sequencing (Life Technologies -Ion Torrent), -Pyrosequencing (Roche - 454), -Sequencing by ligation (Life Technologies - SOLiD), -Reversible terminator - sequence by synthesis (Illumina - Solexa), -Single-molecule real-time DNA sequencing by synthesis (Pacific biosciences - PacBio)	**(Hunt et al., 2015, Pritchard et al., 2012, Metzker, 2010)**
Biosensor Techniques	A biorecognition element (a DNA probe complementary to the miRNA of interest) is in direct contact with a transduction element	-High sensitivity, -Very fast, -Label-free protocols	-Hybridization bias, -Not useful for multiplexing, -Mass transfer challenge, -Reliability of measurements	-Electrochemical -Electromechanical, -Optical-based	**(Johnson and Mutharasan, 2014, Thevenot et al., 2001, Hamidi-Asl et al., 2013, Johnson and Mutharasan, 2012)**
NanoString nCounter	Molecular "barcodes" and microscopic imaging are used to detect and count up to several hundred unique transcripts in one hybridization rea ction	-Multiplex detection, -Direct digital detection, -No need for amplification, -High specificity	-Semi-quantitative, -Not suitable for detection of new molecules, limited dissemination of the instrument, -Need for increased open-source software tools for data analysis.	none	**(Geiss et al., 2008)**

Abbreviations: DIG = digoxigenin, LNA = locked nucleic acids, EDC = 1-ethyl-3-(3-dimethylaminopropyl) Carbodiimide, LED = LNA modified probes, EDC crosslinking and DIG-labeled), DSLE = DIG-labeled, splinted-ligation and EDC cross-linking method, RT-qPCR = reverse transcription - quantitative polymerase chain reaction, SHUT = stacking-hybridized universal tag, PAZ = Piwi/Argonaute/Zwille, RBD = RNA binding protein, LASH ligase-assisted sandwich hybridization

1.7.2 Data processing and molecular integration

The difficulty in correlating miRNA expression with mRNA targets for clinical applications is the fact that miRNAs in physiological and pathological conditions control more than one mRNA (tens, even hundreds) and that one mRNA can be controlled by more than one miRNA **(Rossi and Calin, 2013)**. The major issue in miRNA studies is to identify the targets of the miRNAs and narrow the options to the most important and relevant to the specific case.

Because the *in vitro* and *in vivo* discovery of the real-life targets of miRNAs is a challenging - and time-consuming procedure, web-tools have been designed to tackle this problem and produce lists of the possible targets. The first bioinformatic approach on this subject was developed by Lai in 2002 **(Lai, 2002)**. Lai compared the sequence of a subset of miRNAs to the K box and Brd box motifs that were previously shown to mediate negative post-transcriptional regulation. In this study, it was determined that a region of 8 nucleotides in the beginning of a miRNA displays a perfect complementarity to the motifs, suggesting that this sequence is responsible for the post-transcriptional regulation mediated by miRNAs. This 8 nucleotide sequences is now known as the seed of a miRNA and is the main area responsible for miRNA function. Nowadays the algorithms in the available web-tools that predict possible miRNA targets focus their search on four basic parameters:

1. Seed matching: as mentioned above, the seed matching refers to the complementarity of the first 2-8 nucleotides of a miRNA in the 5'-end to the 3'-UTR of the mRNA target. It is the basic parameter that most (if not all) tools take under consideration. But perfect complementary is not always observed. The main types of seed matching include the 6mer (complementary for 6 nucleotides), 7mer (complementary for 7 nucleotides) and 8mer (perfect complementary) **(Peterson et al., 2014)**.
2. Conservation: the maintenance of the seed region of a miRNA among species enforces the proof of its existence and reduces the number of false positive predictions **(Ritchie et al., 2013)**.
3. Thermodynamic stability – site accessibility: the free energy (Gibbs energy, ΔG) is a measure of the stability of a system. Highly negative ΔG values characterize very stable RNA complexes. By predicting the ΔG between a miRNA and its candidate target it is possible to predict the stability of the duplex and conclude if this complex can form (the higher the ΔG the more stable the complex and more possible the binding of miRNA-mRNA) **(Peterson et al., 2014)**. Also, the energy needed for the unfolding of the secondary structure of the mRNA allowing the accessibility to the miRNA **(Long et al.,**

2007) can be an additional parameter to consider. Less energy needed to unfold the mRNA, the more possible the target site to be accessible **(Peterson et al., 2014)**.

4. Multiple target sites in the 3'-UTR of the mRNA: studies have shown that in the 3-UTR of a mRNA there can be multiple target sites for one miRNA **(Ritchie et al., 2009)**. The different algorithms allow analysis of mRNAs and can predict different possible binding cites for the one miRNA and, thus, increasing the possibility that this miRNA can bind and regulate the mRNA.

Many web-tools are available, each with a different algorithm for calculating the predicted targets of miRNAs **(Peterson et al., 2014).** Complementary to the prediction databases are the databases which contain information on experimentally validated miRNA targets. These databases contain information extracted from citations where a well-documented interaction of a miRNA:mRNA target using dedicated methods (see paragraph below "In vitro target validation" on page 25) **(Lee et al., 2015).** A list of prediction and validation databases is shown in table 2 and 3

Table 2: Available web-tools for miRNA target prediction.

Web-tool Name	Organisms	Calculation Features
miRanda	All	Seed match, conservation, and Thermodynamic stability
miRanda-mirSVR	Humans, rats, mice, flies, and worms	Seed match, conservation, Thermodynamic stability, site accessibility
TargetScan	Mammals, flies, and worms	Seed match and conservation
DIANA-microT-CDS	Humans, mice, flies, and worms	Seed match, conservation, Thermodynamic stability, site accessibility, Multiple target sites
MirTarget2 or miRDB	Humans, mice, rats, dogs, and chickens	Seed match, conservation, Thermodynamic stability, site accessibility
RNA22-GUI	Humans, mice, flies, and worms	Seed match and Thermodynamic stability
TargetMiner	Any	Seed match, conservation, Thermodynamic stability, site accessibility, Multiple target sites
PITA	Humans, mice, flies, and worms	Seed match, conservation, Thermodynamic stability, site accessibility and Multiple target sites
RNAhybrid	Any	Seed match, Thermodynamic stability, Multiple target sites
miRGate	Human, mice, rats	Seed match, conservation, Thermodynamic stability, site accessibility, Multiple target sites
miRWalk 2.0	Human, mice, rats and all transcripts and mitochondrial genomes	Seed match and conservationn

Table 3: Available web-tools for miRNA target validation.

Web-tool Name	Description	Species included
mirRecords	Contains manually curated experimental evidence for 2705 records of interactions between 644 miRNAs and 1901 target genes	9 species including human
StarBase	Large-scale CLIP-Seq data originating from HITS-CLIP, PAR-CLIP, iCLIP and CLASH on more than 6000 samples	14 cancer types (human, mouse, C. elegans)
DIANA – TarBase	High quality manually curated experimentally validated miRNA:gene interactions, enhanced with detailed meta-data.	24 species including human
miRTarBase	Data for experimental miRNA – target interactions collected from literature from reporter assays, western blot, northern blot, RT-qPCR, microarrays, SILAC, NGS	18 species including human
miRWalk 2.0	Experimentally verified miRNA interaction information associated with genes, pathways, organs, diseases, cell lines, OMIM disorders and literature on miRNAs. Around 668.000 interactions	15 species including human
PhenomiR 2.0	Manually curated database with information about differentially regulated miRNA expression in diseases and other biological processes.	Human and mice
miR2Disease Base	A manually curated database that provides a comprehensive resource of miRNA deregulation in various human diseases. Hosts 349 miRNAs related to 163 diseases.	Human

1.7.3 In vitro target validation

Currently, *in vitro* studies are the gold standard for validating predicted miRNA targets. The goal is to monitor any changes of the target gene's expression in response to loss or gain of function of a specific miRNA .This is achieved mainly through artificial manipulation of the miRNA concentration with either the use of double stranded miRNA mimics or miRNA inhibitors (antagomirs). MiRNA mimics are artificial, chemically modified miRNA-like small RNAs with the ability to mimic the function of a miRNA guide strand while bypassing the maturation steps of Drosha and DICER and causing a rapid decrease in the expression of the potential target mRNA **(Sokilde et al., 2015)**. On the other hand, antagomirs act by binding to mature miRNAs, blocking their activity, hence leading to the increased expression of the mRNA target. Figure 9 summarizes the actions of antagomirs and miRNA mimics.

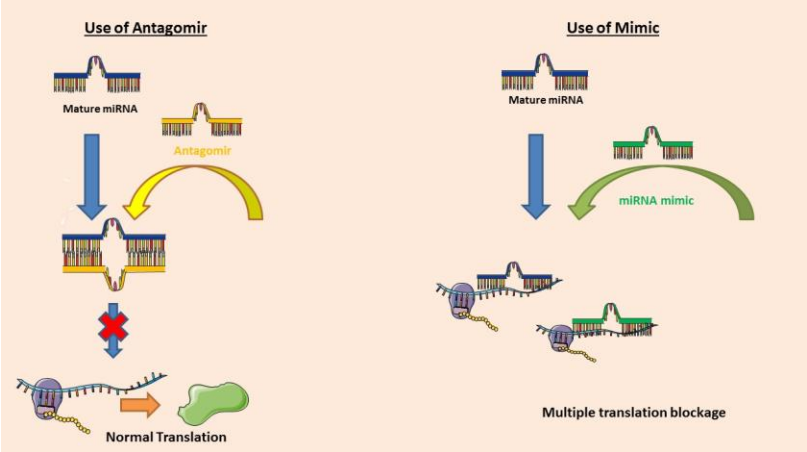


Figure 9: Antagomirs and miRNA mimics. Antagomir function: with the addition of the antagomirs the action of the miRNAs is inhibited. Antagomirs bind in a complementary manner to the miRNAs blocking their action leading to the normal translation of the targeted mRNA. miRNA mimics: the addition of miRNA mimics is leading to additional inhibition of translation of the mRNA - targets molecules.

Quantitative analysis of miRNAs and mRNAs with microarrays or NGS after treating cells with miRNA mimics/inhibitors can provide a panel of the affected mRNAs and thus possible direct targets **(Tarang and Weston, 2014)**. Quantitative proteomic analysis as a high throughput method has also been used to observe the alterations in the level of proteins that are considered possible miRNA targets.

Other popular techniques are the cross-linking immunoprecipitation (CLIP-based) approaches which allow to study the interaction of miRNA-mRNA-AGO protein and, thus, to verify the effect of over or under expression of the miRNAs on their targets with or without the use of inhibitors **(Wen et al., 2011)**. One of the first methods described based on this concept used co-immunoprecipitation of tagged-AGO protein (the main protein of the RISC complex that

connects to the miRNA-mRNA duplex) with the miRNAs and mRNAs **(Karginov et al., 2007, Easow et al., 2007, Hendrickson et al., 2008)**. Two techniques from this category have helped significantly in the identification of the genuine miRNA targets **(Haecker and Renne, 2014)**: High-throughput sequencing of RNA isolated by cross-linking and immunoprecipitation (HITS-CLIP) and photoactivable-ribonucleoside-enhanced cross linking and immunoprecipitation (PAR-CLIP). HITS-CLIP uses UV light to cross-link –freeze- the RISC complex including miRNA-mRNA-AGO2, followed by immunoprecipitation, and sequencing of isolated miRNA and mRNA **(Licatalosi et al., 2008).** PAR-CLIP is a modification of HITS-CLIP where displays more efficient cross-linking and RNA recovery. However, this approach uses live cells that need to be supplemented with the nucleoside analog 4-Thiouridine (4SU) that is incorporated into nascent mRNAs, thus it cannot be applied to tissue samples **(Hafner et al., 2010)**. Both techniques have helped significantly in the identification of the genuine miRNA targets **(Haecker and Renne, 2014)**. **(Danan et al., 2016)**.

However, one of the gold standard target validation method is based on reporter assays. In brief, the potential miRNA target site is cloned and added to the open reading frame of a reporter gene, *e.g.* luciferase of *Renilla* or firefly. The recombinant plasmid is transfected into mammalian cells together with a mimic miRNA. Cells are incubated for 24-48 hours and then the luciferase activity or fluorescence intensity is measured. If the target site of the mRNA is a valid target for the miRNA under study reduced signal intensity compared to control plasmid (transfected with reporter vectors without the UTR sequence, or with a UTR cloned in the antisense orientation) will be observed **(Ritchie et al., 2013).**

A list with the different *in vitro* miRNA target validation methods is available in Table 4.

Table 4: miRNA target validation methods. For the validation phase, miRNA antagomirs or mimics are delivered to an *in vitro* or *in vivo* experimental system to reduce or increase the levels of the miRNAs of interest respectively, following by measuring the levels of the predicted mRNA targets or the protein level originating by the mRNA target.

Method / molecule	Full name	Principle	Can be used for...	Reference
HITS-CLIP	High-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation	UV light to cross-link –freeze- the RISC complex including miRNA-mRNA-AGO2, followed by immunoprecipitation, and sequencing of isolated miRNA and mRNA	*In vitro* – miRNAs and their target mRNAs	**(Licatalosi et al., 2008)**
PAR-CLIP	Photoactivable-ribonucleoside-enhanced cross linking and immunoprecipitation	Similar to HITS-CLIP. Difference: Cells are first supplemented with the nucleoside analog 4-Thiouridine (4SU) that is incorporated into nascent mRNAs.	*In vitro* – miRNAs and their target mRNAs	**(Hafner et al., 2010)**
SILAC	Stable-isotope labeling with amino acids in culture	Relative protein abundance is measured by mass spectrometry of samples labelled with different isotopes	*In vitro* – the level of the protein originating from the mRNA target	**(Ong et al., 2002)**
2D-DIGE	Two dimensional gel electrophoresis	Electrophoresis on a single gel of two samples labelled with different fluorescent dyes, separating the proteins by iso-electric focusing and SDS–PAGE and then identification by mass spectrometry	*In vitro* – the level of the protein originating from the mRNA target	**(Zhu et al., 2007)**
RT-qPCR	Reverse transcription quantitative polymerase chain reaction	Investigate the level of the miRNA or mRNA of interest with TaqMan or Sybr green probes	*In vitro* – the level of the miRNAs and mRNAs	**(Tarang and Weston, 2014)**
Western blot		Electrophoresis to separate proteins followed by staining with antibodies specific to the target protein.	*In vitro* – the level of the proteins predicted to be affected by miRNA deregulation	**(Tarang and Weston, 2014)**
IHC	Immunohistochemistry	*In situ* hybridization with LNA probes complementary against the miRNA of interest and/or immunohistochemical detection of specific proteins	In tissue samples – detect the level of miRNAs and proteins of interest	**(Tarang and Weston, 2014)**

Method / molecule	Full name	Principle	Can be used for...	Reference
Microarrays		Hybridization of a panel of miRNAs or genes to the complementary fixed probes and detection via fluorescence	In all samples examine the expression profile of miRNAs and/or mRNAs	**(Tarang and Weston, 2014)**
RNA Sequencing		Massive parallel sequencing of millions of fragments of DNA from a single sample	In all samples examine the expression profile of miRNAs and/or mRNAs	**(Tarang and Weston, 2014)**
Luciferase assay		A luciferase reporter contains the 3´-UTR target sites of a mRNA target which the miRNA mimics of the miRNA of interest should target and reduce the fluorescence signal	*In vitro* – validation of the miRNA target	**(Ritchie et al., 2013)**
GFP reporter	Green fluorescent protein	A GFP reporter is encoded with multiple 3´-UTR target site of a miRNA and with the presence of the miRNA the expression level of the GFP is lower compared to the absence	*In vitro* and *in vivo* - validation of miRNA target	**(Ritchie et al., 2013)**
Biotin-tagged miRNA		Biotinylated synthetic miRNA is transfected into cells followed by purification of miRNA:mRNA complexes connected to the seed sequence with streptavidin-agarose beads	*In vitro* - capture of miRNA targets	**(Orom and Lund, 2007, Hsu et al., 2009)**
PARE	Parallel analysis of RNA ends	Genome-wide identification of the miRNA-induced cleavage products	*In vitro* and *in vivo* - capture the miRNA-mediated cleavage products	**(German et al., 2008, Addo-Quaye et al., 2008)**
RLM-RACE	RNA ligase mediated-5´ rapid amplification of cDNA ends	The 5´ phosphate of the cleaved, uncapped poly-A RNAs an RNA adapter is ligated followed by reverse transcription. Then the cDNAs are amplified with the adapter and the gene-specific primers to be cloned and sequenced	*In vitro* and *in vivo* - capture the miRNA-mediated cleavage products	**(Llave et al., 2002, Thomson et al., 2011)**

Method / molecule	Full name	Principle	Can be used for...	Reference
LAMP	Labeled miRNA pull-down assay system	The pre-miRNA is labeled with digoxigenin (DIG), mixed with cell extracts, and immunoprecipitated by anti-DIG antiserum	*In vitro* - identification of the target gene of known miRNAs	**(Hsu and Tsai, 2011)**
Reverse transcription of targets		Use of the sequence of the miRNA to generate primers via reverse transcription matching the mRNA targets followed by PCR	*In vitro* - capture miRNA target genes	**(Ritchie et al., 2013)**
Polysome profiling		Cyclohexamide is used to trap elongating ribosomes followed by pull-down of the enclosed mRNA	*In vitro* - detection of the effect of deregulation of a miRNA to the transcriptome	**(Ritchie et al., 2013)**
Ribosome profiling		Cyclohexamide is used to trap elongating ribosomes, cells are lysed and the mRNA enclosed to the monosomes is sequenced	*In vitro* - detection of the effect of deregulation of a miRNA to the transcriptome	**(Ritchie et al., 2013)**

1.7.4 Mimicking or blocking miRNA in vivo

The results from the *in vitro* experiments can be transferred to *in vivo* research with the hope to reproduce a similar outcome. The basic approach is similar *in vitro* and *in vivo*; deliver a synthetic miRNA mimic or inhibitor of a miRNA of interest and then observe the effects on the condition due to the miRNA abundance change or at the protein level with emphasis on the predicted and *in vitro* validated targets. A further step is the observation of any side effects, alterations in neighboring molecular pathways and pathologies and effects on the phenotype. The ultimate goal is to identify a possible therapeutic effect of a miRNA that can pass the clinical trials and enter the clinical practice.

Until now the main focus has been on improving the stability of the molecules interfering with miRNA *in vivo,* since unprotected oligonucleotides can easily be degraded by RNAses in the blood. As a consequence the design of carriers has become essential in order to transfer and deliver the miRNA mimic or antagomir to the desired target while minimizing degradation. These carriers such as nanoparticules, lipid bilayers of viruses (e.g. adeno/lentiviruses) need to have particular features. They need to be large enough to avoid renal and hepatic filtration but also small enough to allow passage through the tissue and cellular barriers and release efficiently their load. Moreover carriers must not activate the immune system and should not be toxic for the organism. Finally, all possible side-effects from the delivery systems and off-target effects must be detected and examined, as, for example, designing an antagomir to inhibit a miRNA of interest and not another with similar sequence. **(Zhang et al., 2013, Chen et al., 2015b, Orom and Lund, 2007)**.

Table 5 summarizes the *in vivo* delivery systems developed so far.

Table 5: miRNA *in vivo* delivery systems

Method / molecule	Principle	Can be used for...	Reference
Direct injection of ant-miRs or miRNA mimics	Direct injection of antagomirs and miRNA mimics into animal model	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Orom and Lund, 2007)**
miRNA sponges or decoys	Single piece of RNA containing multiple seed regions complementary to the miRNA family of interest. By delivering this system into cells, the miRNAs bind strongly to the RNA sequence resulting in silencing the miRNA activity	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Elmen et al., 2008a, Ebert et al., 2007) (Horie et al., 2009)**
Modified viruses	Lentivirus, adenovirus and adeno-associated virus are modified accordingly to transfer and deliver miRNA regulators in a model system	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Chistiakov et al., 2012, Chen et al., 2015b)**
Exosomes	Microvesicles that naturally transfer miRNAs and can be used to deliver modified miRNA regulators	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Zhang et al., 2010)**
Liposomes (DOTMA, SLNs, MaxSuppressor In Vivo RNA-LANCEr II, LPH, scFv, iNOPs)	Lipid bilayers (cationic or neutral) with an internal aqueous phase to load and carry the desired molecules	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Wu et al., 2011, Hsu et al., 2013, Liu et al., 2015, Wiggins et al., 2010)** **(Trang et al., 2011, Chen et al., 2010) (Su et al., 2011)**

Method / molecule	Principle	Can be used for...	Reference
PLGA (poly(lactide-co-glycolic acid)	PLGA polymers are transformed into microparticles or nanoparticles enclosing biological molecules that can release via endolysosomal escape after entering the cell	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Kulkarni et al., 1971, Chen et al., 2015b, Zhang et al., 2013) (Panyam and Labhasetwar, 2003, Blum and Saltzman, 2008)**
PEI (polyethylenimine)	Cargo release follows the so-called "proton sponge effect"; once the polymer interacts with the surface of the cell, endocytosis takes place. The PEI causes endosome swelling by influx of protons and water (proton sponge effect) which leads to endosome destabilization and the release of the miRNA	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Zhang et al., 2013, Boussif et al., 1995)**
Inorganic nanoparticles (Gold nanoparticles, disialoganglioside antibody) (AUNP-S-PEG, GD2)	Inorganic nanoparticles are bound and deliver miRNA regulators	*In vitro* - *in vivo* antagomir or miRNA mimic system and detection/observation of the expression level of the possible targets and/or physiology	**(Chen et al., 2015b, Zhang et al., 2013) (Ghosh et al., 2013)**

1.8. miRNAs as potential therapeutic agents and biomarkers: lessons learned so far

1.8.1. Therapeutic potential

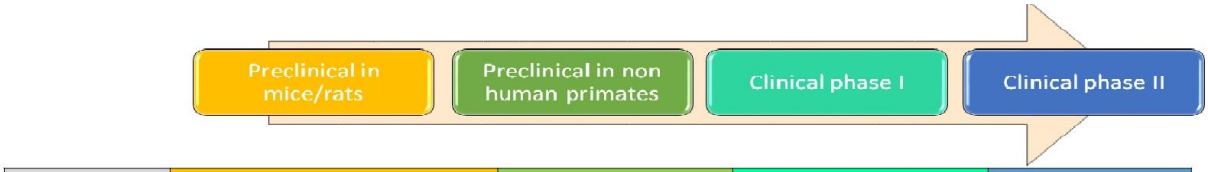
The most extensively studied miRNA to be potentially introduced in clinical practice is miRNA-122, an abundant liver-specific miRNA with a key role in liver function (fatty acid and cholesterol metabolism) and hepatitis C (HCV) progression **(Takata et al., 2013)**. Elmen et al. showed that reduction of miRNA-122 with LNA-ant-mi-R122 (antagomir for miRNA-122) led to reduction of cholesterol levels in plasma of mice and non-human primates **(Elmen et al., 2008b, Elmen et al., 2008a)**. Lanford et al. showed that suppression of HCV viremia by an antagomir for miR-122 in chimpanzees lead to improvement HCV-liver pathology **(Lanford et al., 2010)**. In these three studies lasting from 3 weeks to 3 months, no short-term toxicity was observed, opening the way for use of the miRNA-122 antagomir as a drug in liver disease. Indeed, these preclinical results led to the development of miravirsen, the first antagomir drug candidate against HCV by Santaris Pharma A/S **(Janssen et al., 2013)**. Miravirsen binds to the stem-loop structure of pri- and pre-miR-122 with nanomolar affinity, and inhibits both Dicer- and Drosha-mediated processing of miR-122 precursors and has entered Phase II trials, after displaying no side effects on healthy volunteers **(Gebert et al., 2014)**.

Another therapeutic miRNA candidate that is now in Phase I trials is the liposome-formulated mimic miRNA-34, or MRX34 as it is named by the developing company MiRNA Therapeutics. The miRNA-34 family is found to be decreased in many cancer types. It was observed that the miRNA-34 family can regulate the p53 pathway by direct inhibition of many oncogenes including Myc, c-Met, BCL-2, CDK4 and CDK6. Treatment with miR-34 mimics reduced tumor-size or -growth in many cancer model systems **(He et al., 2007, Li et al., 2015a, Liu et al., 2011, Roy et al., 2012)**. MRX34 could become one of the first mimic miRNA systems to restore the level of a miRNA and show significant anti-tumor activity (http://www.mirnatherapeutics.com/pipeline/mirna-pipeline.html).

Another company, Miragen therapeutics, has initiated several preclinical studies for blocking a number of miRNAs. MiR-155 is studied in hematological malignancies and amyotrophic lateral sclerosis and miR-29 in cutaneous and pulmonary fibrosis. Antagomirs against miR-155 and miR-29 have passed the pre-clinical study stage and are now entering clinical trials. Moreover, Miragen therapeutics is performing preclinical studies in heart failure targeting miR-208 and miR-15 and in peripheral artery disease targeting miR-92 (http://miragentherapeutics.com/pipeline/).

Finally, Regulus Therapeutics has initiated their ant-miR programs for blocking of miR-122 in HCV and miR-10b in glioblastoma. In addition, in non-human primates, tests with an ant-miR-33a/b showed positive results against atherosclerosis. In collaboration with Sanofi, Regulus is developing an ant-miR-221 against hepatocellular carcinoma and an ant-miR-21 for kidney fibrosis, the leading complication in kidney disease, and with AstraZeneca an ant-miR-103/107 for the treatment of non alcoholic steatohepatitis (NASH) in patients with type 2 diabetes/pre-diabetes (http://www.regulusrx.com/therapeutic-areas).

Figure 10 summarizes the aforementioned studies.



MIRNA THERAPEUTICS	Mimic - Let-7a Mimic - miR-16 (cancer)	Mimic - miR-215 Mimic - miR-101 (cancer)	Mimic - miR-34 family (cancer)	
REGULUS THERAPEUTICS	ant-miR-10b (glioblastoma) ant-miR-221 (hepatocellular carcinoma) ant-miR-21 (kidney fibrosis) ant-miR-103/107 (Insulin resistance)	ant-miR-33a ant-miR-33b (atherosclerosis and metabolic syndromes)	ant-miR-122 (HCV)	
MIRAGEN THERAPEUTICS	ant-miR-155 (hematological malignancies and amyotrophic lateral sclerosis) ant-miR-29 (cutaneous and pulmonary fibrosis) ant-miR-92 (peripheral artery disease) ant-miR-208 ant-miR-15 (heart failure)			
SANTARIS PHARMA				ant-miR-122 (HCV)

Figure 10: miRNAs being considered for clinical use by Industry and their progression in (pre)clinical trials.

1.8.2. miRNAs as potential biomarkers

Another application is the use of miRNAs as potential diagnostic and prognostic biomarkers. A search in PubMed with the words "miRNA" AND "Biomarker" returns more than 5000 publications, the majority related to cancer. However, as in miRNAs in therapy, further validation of these miRNAs as potential biomarkers of disease is necessary before a potential clinical application. As such, almost all reports provide strong indications of the possible use of the miRNAs as diagnostic and/or prognostic biomarkers. Below are discussed some key studies on the subject with emphasis on human studies.

1.8.2.1 Tissue miRNAs - cancer

In 2002, the first miRNAs characterized as being downregulated in patients with B-cell chronic lymphocytic leukemia were miR-15a and miR-16-1 **(Calin et al., 2002)**, a year after the discovery of the second miRNA, let-7. Later on, the miR-17-92 cluster was identified to be upregulated in many kinds of cancers (mostly in lymphoma and leukemia) and associated with upregulation of the oncogene c-Myc, providing a first well characterized cancer associated miRNA cluster and introducing a new class of miRNAs, the oncomiRs **(He et al., 2005, Dews et al., 2006, Lu et al., 2007)**. Additional miRNAs including miR-21 **(Medina et al., 2010)**, miR-34 family **(Bommer et al., 2007, He et al., 2007)** and let-7 **(Johnson et al., 2005, Esquela-Kerscher et al., 2008)** are by now accepted as cancer associated miRNAs.

The first key report that opened the road for considering the miRNAs as possible biomarkers came from the laboratory of Todd Golub covering 217 mammalian miRNAs and several hundred samples, including clinical samples, common cancer cell lines and mouse tumors. The authors demonstrated that miRNA profiles could distinguish a tumor's developmental origin and that miRNAs are generally downregulated in cancers (129 out of 217 miRNAs). In addition, they noticed that poorly differentiated tumors displayed lower miRNA expression compared to tumors with a higher differentiation **(Lu et al., 2005)**. The latter was confirmed by Kumar *et al.* by performing experiments of conditional knockout mice for Dicer crossed with a K-Ras driven lung cancer model and demonstrating that a global reduction of miRNA biogenesis leads to reduced survival in affected mice and therefore may play tumor suppressive properties **(Kumar et al., 2009)**.

As the years were passing technology evolved allowing more routine screening of miRNAs profiles in disease. Specific miRNA signatures allowed the definition of subtypes of

cancers, such as the use of a panel of 133 miRNAs from breast tumor samples to distinct between basal and luminal breast cancers **(Blenkiron et al., 2007)**. In their review Omar Faruq and Andrea Vecchione discuss extensively the variety of miRNA signatures to define tumor subgroups **(Faruq and Vecchione, 2015)**. Table 6 summarizes the most important miRNAs signatures in cancer.

miRNAs were also reported to have potential clinical prognostic value. The first report on that subject came from Takamizawa *et al.* showing that decrease of let-7a was correlated with poor survival in lung cancer patients **(Takamizawa et al., 2004)** and a year later another important report mentions a panel of 13 miRNAs as prognostic biomarkers in chronic lymphocytic leukemia (CLL) **(Calin et al., 2005)**. Of all miRNAs, miR-21 (increased in cancer) and let-7 (decreased in cancer) were the most common miRNAs associated with patient outcome **(Hayes et al., 2014)**. The oncogenic miR-21 is overexpressed in many cancers, including breast cancer, glioblastoma, hepatocellular carcinoma, lung cancer, stomach cancer, colorectal cancer, and prostate cancer **(Pan et al., 2010)**. The mechanistic function of miR-21 is to create a pro-tumorigenic environment by targeting numerous tumor suppressor genes with roles in apoptosis, invasion, and proliferation, and thus is considered as number one novel molecular target for cancer therapy **(Pan et al., 2010)**.

Table 6: miRNAs that can differentiate tumor subgroups. Table adjusted from **(Faruq and Vecchione, 2015)**

Cancer	Specific miRNA	Sources	Expression	Significance
Breast cancer	miR-342	Tissue	Up	Luminal B-like breast tumors vs. healthy controls
	miR-18a, miR-135b, miR-93, miR-155	Different	Up/Down	Basal-like vs. healthy controls
	miR-145, miR-99a, miR-100, miR-130	Different	Up/Down	Normal-like vs. healthy controls
Renal cell carcinoma	miR-195, miR-15b, miR-99a, miR-191	Tissue	Up	ccRCC vs. other subtypes
	miR-200b	Tissue	Up	Chromophobe RCC (chRCC) vs. oncocytoma
	miR-211	Tissue	Up	chRCC and oncocytoma vs. ccRCC and pRCC
	miR-210	Tissue	Up	Conventional RCC (cRCC) vs. healthy controls
Lung Cancer	miR-21, miR-29b	Tissue	Up	NSCLC vs. SCLC
	miR-21, miR-126, miR-210, miR-182	Serum	Up/Down	Diagnosis of stage-I NSCLC – lung adenocarcinoma vs. squamous cell cancinoma
	miR-205	Tissue	Up	Squamous vs. non-squamous NSCLC
Pancreatic Cancer	mR-375	Plasma	Up	(PDAC) vs. others pancreatic and gastric carcinoma
DLBCL	miR-21, miR-155 and miR-221	Different	Up	ABC-DLBCL vs. GCB-DLBCL subtypes
	miR-10393-3p	Tissue	Up	Diagnosis of DLBL patients
	miR-10397-5p	Tissue	Up	ABC-DLBCL vs. other DLBC
	miR-5586-5p	Tissue	Up	GCB-DLBCL vs. ABC-DLBCL and unclassified DLBCL
	miR-30b	Tissue	Down	GCB-DLBCL vs. other DLBCL
	miR-515-3p, miR-155, miR-598, miR-625, miR-199a-5p	Tissue	Up/Down	PTCL-NOS vs. ALCL/ALK-
	miR-652, miR-627, miR-519e, miR-487b, miR-324-5p, miR-449a, miR-381, miR-574-3p	Tissue	Up/Down	PTCL-NOS vs. AITL
	miR-124, miR-325, miR-181a, miR-618	Tissue	Up/Down	ALCL/ALK− vs. ALCL/ALK+
PTCL	miR-512-3p, miR-886-5p, miR-886-3p, miR-708, miR-135b	Tissue	Up	Diagnosis of ALCL/ALK+ disease vs. ALCL/ALK− + other PTCLs
	miR-146a, miR-155	Tissue	Down	
	miR-210, miR-197, miR-191, miR-512-3p	Tissue	Up	Diagnosis of ALCL/ALK− vs. ALCL/ALK+ and other PTCLs

Abbreviations: ccRCC, clear cell renal carcinoma; chRCC, chromophobe renal cell carcinoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; DLBCL, diffuse large B-cell lymphoma; ABC, activated B cell-like; GCB, germinal center B cell like; PTCL, peripheral T-cell lymphoma; PTCL-NOS, PTCL-not otherwise specified; AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large-cell lymphoma; ALCL/ALK+, ALCL− anaplastic lymphoma kinase present; ALCL/ALK−, ALCL− anaplastic lymphoma kinase absent.

1.8.2.2. Circulating miRNAs – cancer

The vast majority of miRNA expression profile reports in cancer stem from solid tissues. However, the high stability of the miRNAs allows them to be readily detected in human plasma, serum or total blood. Early studies on circulating miR-21 in serum in patients with diffuse large B-cell lymphoma, highlighted the potential of circulating miRNAs as biomarkers **(Lawrie et al., 2008)**. In 2008 Markou *et al.* published for the first time evidence for the prognostic value of the miRNAs in a clinical study on lung cancer. The authors characterized the overexpression of miR-21 in patients with lung cancer as a negative prognostic factor **(Markou et al., 2014)**. Three years later, an important study from Boeri *et al.* also, demonstrated that circulating miRNAs can produce a miRNA signature with strong predictive value in lung cancer patients years before the onset of disease by analyzing expression in samples taken before diagnosis, at the time of detection by computed tomography and in disease-free smokers **(Boeri et al., 2011)**. An important study by Keller *et* involved the analysis of 863 miRNAs in 454 human blood samples from patients suffering from 14 separate diseases including lung cancer, prostate cancer, pancreatic ductal adenocarcinoma, melanoma, ovarian cancer, gastric tumors, Wilms tumor, pancreatic tumors, multiple sclerosis, chronic obstructive pulmonary disease, sarcoidosis, periodontitis, pancreatitis and myocardial infarction. In that study, on average, more than 100 miRNAs were found deregulated in the blood for each different disease. By utilizing this data and developing mathematical algorithms and probability plots the authors were able to accurately predict the disease in more than two thirds of individuals involved in the study **(Keller et al., 2011)**.

1.8.2.3. Clinical miRNA diagnostics - cancer

The closest potential of miRNAs use in current diagnostics started with a study comparing miRNA expression, using miRNA microarrays, in tissue of 205 primary versus 131 metastatic tumors covering 22 different tumor origins. Rosenfeld *et al.* developed a binary decision tree classifier based on miRNA expression with tissues displaying the highest specificity of certain miRNAs placed at the top of the tree. The result of this classifier was that a low number of miRNAs (n=48) could predict tumor origin with a 90% accuracy when tested on a blinded set **(Rosenfeld et al., 2008)**. This study, together with other studies which followed with the same concept confirmed the accuracy of using miRNAs as diagnostics for tumors of unknown origin **(Varadhachary et al., 2011, Mueller et al., 2011)**, were partly driven by the miRNA diagnostics company Rosetta Genomics, Israel. Rosetta Genomics is now offering a panel (miRview-mets2) to clinicians to determine the origin of metastatic

cancers when the primary origin of metastasis is questionable. The panel consists of a test of 64 miRNA biomarkers validated on 489 samples of which 146 represent metastatic tumors covering 42 tissues of origin **(Meiri et al., 2012)**. In addition to the "miRview-mets2" panel, Rosetta Genomics also offers four additional clinical tests: "miRview-lung", "miRview-squamous", "miRview-meso" and "miRview-kidney". MiRview-lung differentiates four types of primary lung cancer (small cell lung cancer, squamous non-small cell lung cancer (NSCLC), non-squamous NSCLC and carcinoid) using eight separate miRNA biomarkers **(Gilad et al., 2012)**. MiRview-squamous separates non-small cell lung cancers (NSCLC) into squamous cell carcinomas and adenocarcinomas using only miR-205 **(Lebanony et al., 2009)**. MiRview-meso defines samples into a mesothelioma or nonmesothelioma origin based on three separate miRNAs **(Benjamin et al., 2010)** and miRview-kidney separates kidney cancer into its four primary types (benign oncocytoma, clear cell renal carcinoma, papillary renal carcinoma and chromophobe renal carcinoma) using six miRNAs **(Fridman et al., 2010)**.

The move of miRNA diagnostics into the clinics led by Rosetta Genomics and others may aid the use of personalized medicine in the treatment of cancers. However, miRNA diagnostics are still far from entering the clinical routine and their use is focusing mainly on cancer. Hayes *et al.* discuss in their review on the diagnostic value of miRNAs in cancer a list of miRNAs which are now in clinical trials for biomarkers **(Hayes et al., 2014)** (Table 7).

Table 7: miRNA studies that are under clinical trials for use as biomarkers in different cancer types. Table from **(Hayes et al., 2014)**. (Numerous=miRNAs from different sample origins, Circulating=only miRNAs from blood)

MicroRNA	Trial reference	Disease	Trial
Numerous	NCT01964508	Thyroid cancer	Observational study of microRNA expression in fine needle aspirates
Numerous	NCT01722851	Breast cancer	Observational studies of microRNA expression levels as biomarkers of response to treatment in the tumors and circulation
Numerous	NCT01722851	High-risk prostate cancer	Observational studies of microRNA expression levels as biomarkers of response to treatment in the tumors
miR-10b	NCT01849952	Glioma	Observational studies of miR-10b expression levels as biomarkers of tumor grade, survival, and genotypic variation
miR-29b	NCT02009852	Oral squamous cell carcinoma	Observational study to explore the prognostic value of miR-29b in tissue, blood, and saliva
Numerous	NCT02127073	Breast cancer	Interventional study employing intranasal oxytocin to increase the volume of nipple aspirate fluid for biomarker identification and subsequent microRNA profiling of this aspirate
Numerous	NCT01595139	Low-grade glioma	Observational study of the circulating microRNA expression patterns in low grade glioma as early predictors of cancer and as a marker of response to therapy
Numerous	NCT01828918	Colorectal carcinoma	Observational study identifying biomarkers for patient stratification in tissue samples
Numerous	NCT01119573	Endometrial cancer	Observational study of association of microRNA expression and lymph node metastasis in tissue samples
Numerous	NCT01595126	Central nervous system cancer	Observational study of microRNA expression in the blood, cerebrospinal fluid and urine of patients through the course of their treatment
miR-29 family	NCT01927354	Head and neck squamous cell carcinoma	Observational study to investigate the role of microRNA in Twist1-mediated cancer metastasis
Numerous	NCT01453465	Rhabdoid tumors	Observational study to identify microRNA expression patterns between rhabdoid tumors of the kidney and atypical teratoid rhabdoid tumors
Numerous	NCT01391351	Ovarian cancer	Observational studies of biomarker of response to treatment
Numerous	NCT01505699	B-cell acute lymphocytic leukemia	Observational studies of biomarkers of clinical outcomes
Numerous	NCT01957332	Breast cancer	Observational study correlating microRNA expression patterns with imaging and clinical data
Numerous	NCT01556178	Pediatric brain cancer	Observational study of microRNAs in the blood and cerebrospinal fluid as biomarkers
Numerous	NCT01050296	Pediatric solid tumors	Observational study of microRNA expression profiles in different tumor types
Numerous	NCT00864266	Non-small-cell lung cancer	Interventional study to identify a signature of response to chemotherapy
Numerous	NCT01108159	Hematologic cancer	Observational studies of biomarker of expression profiles in initiation, progression and treatment response

1.10. Conclusion

miRNAs have a key role in maintaining cellular homeostasis by regulating many biological functions. They have been studied under many pathological conditions to exploit their potential as biomarkers or therapeutic targets of disease. The early output of miRNA research is encouraging. However, some limitations of using miRNAs in the clinical practice should be mentioned. First, little is known about the phenotypical consequences of miRNA targeting when the results are transferred from the *in vitro* to the *in vivo* setting. In addition, the multiple-targets of miRNAs and the regulation of a single mRNA from a variety of miRNAs render the targeting therapy difficult to monitor, eg the off-target side effects **(Tarang and Weston, 2014).** Moreover, the lack of *in vivo* validation can be due to the lack of robust and efficient miR target prediction tools. Development of effective bioinformatics analysis methods should lead to confident miRNA-mRNA interaction results. Therefore a more integrated collaboration between clinical and molecular methods with a strong biostatistical and bioinformatics foundation to bridge the gap between research and clinical applications and increase the possibilities for validating miRNAs as important biomarkers or drug targets is needed **(Hunt et al., 2015)**. As technology is evolving, new and exciting information will be added to the miRNA biology research; pathway regulations will be revealed and allowed to be manipulated potentially allowing entry of miRNAs in personalized medicine.

In this thesis, we are investigating the role of miRNAs in two major kidney pathologies: Chronic kidney disease in adults and obstructive nephropathy in children.

2. (CHRONIC) KIDNEY DISEASE and miRNAs

2.1. The kidney

The kidneys are bean-shaped organs with the size of a fist and are located below the rib cage near the middle of the back (Figure 11). Their main function is to filter around 180 L of plasma every day in order to remove the waste products from the activities of the body before they become toxic. In addition, kidneys secure the right amount of water and electrolytes in the body, like sodium, potassium and phosphorous **(Chmielewski, 1992)**.

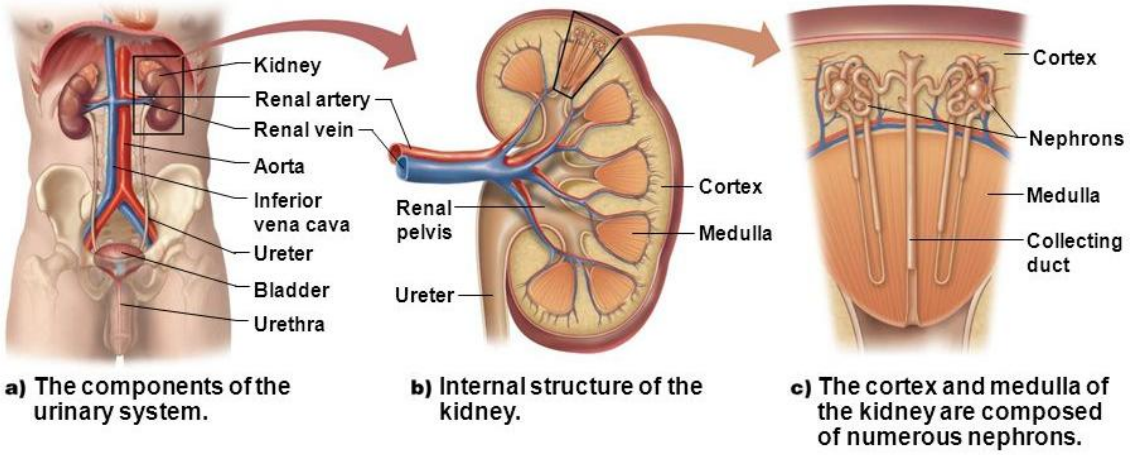


Figure 11: Location, macro and micro structure of the kidney (taken from Pearson Education Inc.).

The gross structure of the kidney consists of two major parts: the renal cortex and the renal medulla (Figure 11). Along the cortex and medulla are spread over 1 million multicellular functional units, so-called nephrons. The main blood filtration section of the nephron is the glomerulus to produce the primary urine. This primary urine is further concentrated in the tubuli, where valuable minerals, proteins, etc, are also resorbed and/or secreted **(Preuss, 1993)**.

The kidneys also contribute to the homeostasis of the body by releasing among other compounds erythropoietin (stimulates the production of red blood cells in the bone marrow), renin (for the stability of blood pressure) and calcitriol (a form of vitamin D - helps to the maintenance of the bones and calcium level in the body) **(Chmielewski, 2003)**.

2.2. miRNAs in kidney physiology

A number of miRNAs have been characterized as kidney specific (miR-215, miR-146a and miR-886) or enriched in kidney as well as other organs (miR-192, miR-194, miR-21, miR-200a, miR-204, let-7a-g) **(Baskerville and Bartel, 2005, Sun et al., 2004, Landgraf et al., 2007)**. But, the evidence of the significance of miRNAs in kidney development and normal function derive from studies on Dicer. Deletion of Dicer has been associated with premature termination of nephrogenesis, formation of tubular and glomerular cysts, disruption of podocyte homeostasis causing dysfunctions at the glomerular filtration barrier leading to glomerulopathy and glomerular and tubulointerstitial fibrosis and deregulation of the control of the blood pressure, reduced rennin concentration, hypertension, kidney fibrosis and disruption of electrolyte homeostasis **(Nagalakshmi et al., 2011, Shi et al., 2008, Patel et al., 2012, Harvey et al., 2008, Ho et al., 2008, Sequeira-Lopez et al., 2010)**.

Specific miRNAs with involvement in kidney development are:

- miR-17~92 cluster: depletion leads to reduced numbers of nephrons, renal hypodysplasia, glomerular dysfunction and proteinuria **(Marrone et al., 2014)**
- miR-192: part of electrolyte homeostasis. Suppresses the Na^+/K^+ ATPase β1 subunit gene in human renal epithelial cells and inhibits the serine/threonine kinase WNK1 in the distal nephrons **(Mladinov et al., 2013, Elvira-Matelot et al., 2010)**
- miR-802 and miR-194: they regulate the renal outer medullary potassium channel (ROMK) by targetin caveolin-1 (a negative regulator of the channel) and intersectin-1 (involved in mediating the WNK-induced endocytosis of the ROMK channel) **(Lin et al., 2011, Lin et al., 2014)**
- miR-9 and miR-374: regulate the expression of claudin-14 (critical for Ca^+ reabsorption in the kidney) in the thick ascending limb of Henke **(Gong et al., 2012)**

2.3. Chronic Kidney Disease

The KDIGO guideline defines CKD as abnormalities of kidney structure or function, present for more than 3 months, with implications for health **(Stevens et al., 2013)**. It is one of the major public health problems worldwide. CKD and its final stage (end stage renal disease, ESRD) represent a huge burden. According to Global Burden of Disease study in 2010, CKD recorded an increase in absolute number of deaths by 82% from 1990 to 2010 climbing from 27th to 18th place as cause of death, setting CKD to the top three fastest growing main causes of death worldwide behind HIV and diabetes **(De Nicola and Minutolo, 2016, Lozano et al., 2012)**. Age-standardized death rates from CKD increased by 15% in this period, while rates for most diseases fell, including other non-transmissible diseases such as major vascular diseases, chronic pulmonary disorders, most forms of cancer and liver cirrhosis. Interestingly, it was observed in that study that for most non-transmissible causes of death, the global increase in the number of deaths was due to ageing of the population, while for CKD both ageing of the population and ageing-independent factors were contributing **(Lozano et al., 2012)**. Age-independent CKD risk factors include sex, obesity, smoking and high cholesterol and triglyceride levels **(Ortiz, 2015, White et al., 2005)**. CKD is associated with an increased risk of dying from cardiovascular causes (including atherosclerosis, arrhythmia, etc), but also from non-cardiovascular diseases including pulmonary embolism, infection and cancer **(James et al., 2008, Thompson et al., 2015, Kurella et al., 2005)**.

CKD is divided into 5 stages. These stages are mainly based on measured or estimated GFR (Figure 12). There are five stages but kidney function is normal in Stage 1, and minimally reduced in Stage 2.

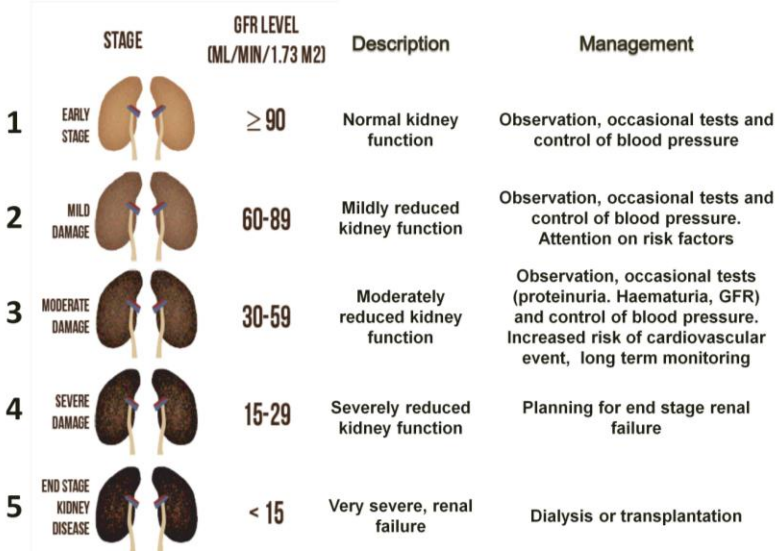


Figure 12: The stages of CKD according to eGFR measurements.

2.4. Known mechanisms in CKD and involvement of miRNAs

2.4.1. Inflammation

Inflammation is a natural mechanism of defense enabling the removal of inciting agent and initiates the healing process as response to injury, infection, ischemia and autoimmune diseases **(Gabay and Kushner, 1999, Kimmel et al., 1998)**. The damaged cells and immune cells (which are close to the injury) mediate a number of substances to activate a response common to several tissue types **(Gerard and Rollins, 2001, Tracey, 2002, Moss et al., 2004)**. Activation of the immune system due to tissue damage, with consequently enhanced inflammation, may facilitate disease onset and progression **(Gerard and Rollins, 2001).** An increased inflammatory response leads to loss of peripheral tolerance to the components of the tissues themselves, which become antigenic and develop local inflammation **(Moss et al., 2004)**. Inflammatory mediators act on target cells, which may be localized in any body compartment, thus leading to different morbid processes, such as CKD **(Moss et al., 2004, El Nahas, 2005).**

Regarding renal tissue, inflammation actively participates in the mechanisms of renal damage progression in diseases of several etiologies with some key events occurring to include 1) increased production of angiotensin II and promotion of inflammatory mediators (cytokines and chemokines) due to glomerular proteinuria,; 2) initial neutrophyl recruitment followed by by macrophages and T lymphocytes recruitment, which unleash the immune response, producing interstitial nephritis; 3) tubular cells respond to this inflammatory process by changes in their phenotype (potentially epithelial-mesenchymal transition) that might lead to transformation of tubular cells into interstitial (myo)fibroblasts,; 4) (myo)fibrobrasts producing collagen which, in turn, injuries the renal vessels and tubules, eventually generating an acellular scar **(Vianna et al., 2011)**.

Toll-like receptors and nuclear facter-kβ (NF-kB) pathways are associated as inflammation mechanisms by controlling necrosis mechanisms and activating proinflammatory responses **(Backdahl et al., 2009, McCall et al., 2011)**.

Many miRNAs have been recognized to have a key role in inflammation response. Some of the most studied are miR-181a and miR-223 on establishing and maintaining the cell fate of immune cells, miR-146 for being involved in innate immunity by regulating Toll-like receptor signaling and ensuing cytokine response, miR-155 on the regulation of the central elements of the adaptive immune response such as antigen presentation. miR-181a is also responsible for T cell receptor signaling while alterations on miR-203 and miR-146 expression in chronic inflammatory diseases suggests their involvement in immune-mediated diseases **(Sonkoly et al., 2008, Ichii et al., 2012)**. Finally a high-throughput miRNA sequencing of peripheral blood mononuclear cells from ten clinically stable hemodialysis patients and ten healthy controls identified 182 differentially expressed miRNAs (including miR-21, miR-26b, miR-146b, miR-155) and correlated their dysregulation with inflammation **(Zawada et al., 2014)**.

2.4.2. *Epithelial Mesenchymal Transition (EMT)*

A molecular reprogramming of the cell is responsible for the differentiation of the epithelial cell to assume the phenotype of matrix-producing fibroblasts and myofibroblasts. As CKD progresses, the tubular epithelial cells at the site of injury start to transform to fibroblasts (more than 30% of the fibroblasts) via the EMT process, **(Iwano et al., 2002)**. In favour of the activation of the EMT process are various profibrotic growth factors including transforming growth factor (TGF), fibroblast growth factor-2, connective tissue growth factor, and angiotensin II **(Kalluri and Weinberg, 2009)**. In response to these autocrine and/or paracrine signals, the tubular cells undergo down-regulation of their epithelial and up-regulation of a mesenchymal genetic program, resulting in loss of epithelial proteins such as E-cadherin, zonula occludens-1, and cytokeratin, and acquisition of mesenchymal markers such as vimentin, matrix metallopeptidase-2, fibroblast-specific protein-1, type I collagen, and fibronectin **(Kalluri and Weinberg, 2009, Zeisberg and Neilson, 2009)**. Additional alterations of the cells include phenotypic changes, loss of epithelial cell adhesion and polarity, actin reorganization and α-smooth muscle actin (SMA) expression, tubular basement membrane disruption, and increased cell migration and invasion **(Liu, 2004)**.

One miRNA has been well studied to have an energetic role in EMT. miR-200 has been shown to regulate negatively EMT by targeting ZEB1 and ZEB2, two important transcriptional repressors of genes regulating cell adherence (E-cadherin) and polarity (CRB3 and LGL2) **(Davalos et al., 2012)**.

2.4.3. *Renal Fibrosis*

TGF-β expression and activity is key in the development of fibrosis, a hallmark of CKD **(Liu, 2004)**. Various stimuli, including high glucose, angiotensin II and reactive oxygen species trigger a response from distressed renal cells and inflammatory cells to produce TGF-β and, thus, promoting renal fibrogenesis and pathological events in the glomerular, tubulointerstitial and vascular compartments. TGF-β is responsible for mesangial cell hypertrophy and proliferation, podocyte apoptosis and detachment from the glomerular basement membrane and endothelial-to mechenchymal transition **(Trionfini et al., 2015)**. This molecule acts by activating the type I TGF-β receptor by binding to type II TGF-β receptor. Type I TGF-β receptor phosphorylates receptor-regulated Smad2 and Smad3, which binds to Smad4 and translocates to the nucleus to bind and activate its target genes **(Meng et al., 2013)**.

miRNAs have been studied in the context of fibrosis. miR-192 is positively regulated by TGF-β to promote fibrosis in the mouse model of unilateral ureteral obstruction and in the rat model of remnant kidney disease. Knocking down miR-192 in these animal models prevents TGF-β–mediated collagen matrix expression **(Chung et al., 2010)**. Another profibrotic, and mostly documented miRNA related to kidney fibrosis, is miR-21 which is, also, positively regulated by TGF-β to target Smad7, a TGF-β suppressor, resulting in promoting fibrosis **(Trionfini et al., 2015)**. miR-21 is over-expressed

both in animal models of kidney disease and human acute and/or chronic kidney disease **(Markou et al., 2014)**. In particular, miR-21 is up regulated in kidney tissue of patients with diabetic nephropathy targeting PTEN and SMAD7 resulting to renal fibrosis progression **(Boeri et al., 2011)**, and also up regulated in kidney tissue of patients with IgA nephropathy associated to activation of fibrogenesis in podocytes and tubular cells **(Keller et al., 2011)**. Interestingly, Gomez *et al.* recently presented the potential therapeutic value of miRNA silencing in Alport nephropathy, a genetic disease characterized by glomerulonephritis leading to end stage CKD in early adult years. In particular, they injected antagomir against miR-21 (ant-miR-21) in a Col4a3$^{-/-}$ mouse model to observe that compared to the control model, ant-miR-21 prevented from glomerulosclerosis, tubular atrophy and interstitial fibrosis, as well as preserving the number of podocytes. Furthermore, the authors showed reduced tubular injury, inflammation and myofibroblastes and, in total, improvement of the kidney function, disease pathology and life span of the treated mice compared to the non treated Col4a3$^{-/-}$ mice. They concluded that up-regulation of miR-21 plays an important role in the development of Alport nephropathy and targeting miR-21 with antagomirs is an effective treatment, leaving promises for the use of miRNA inhibitors (or mimics) as potential therapeutic methods for kidney pathologies **(Gomez et al., 2015)**.

The role of the protector against fibrosis is awarded to miR-29. Qin et al showed that wild-type mice with obstructive nephropathy had reduced expression of miR-29 and progressive renal fibrosis, whereas Smad3 knockout mice had up-regulation of miR-29 without renal fibrosis **(Qin et al., 2011)**. *In vitro* experiments in renal tubular cells showed that miR-29b overexpression inhibited TGF-β up-regulation of collagens I and III. Smad7 also is involved in controlling the TGF-β/Smad3-regulated miRNAs, and its overexpression protects the kidney from renal fibrosis by suppressing expression of miR-192 and miR-21, and restoring expression of miR-29b **(Chung et al., 2013)**.

2.5. Major etiologies responsible for of CKD

The leading cause of CKD is diabetic kidney disease as result of increased cases of diabetes which is estimated to affect globally more than 285 million adults aged 20 - 70 years **(Ayodele and Alebiosu, 2010)**. The second cause is hypertensive kidney disease. Other frequent etiologies include chronic glomerulonephritis, chronic interstitial nephritis and renovascular disease (Figure 13).

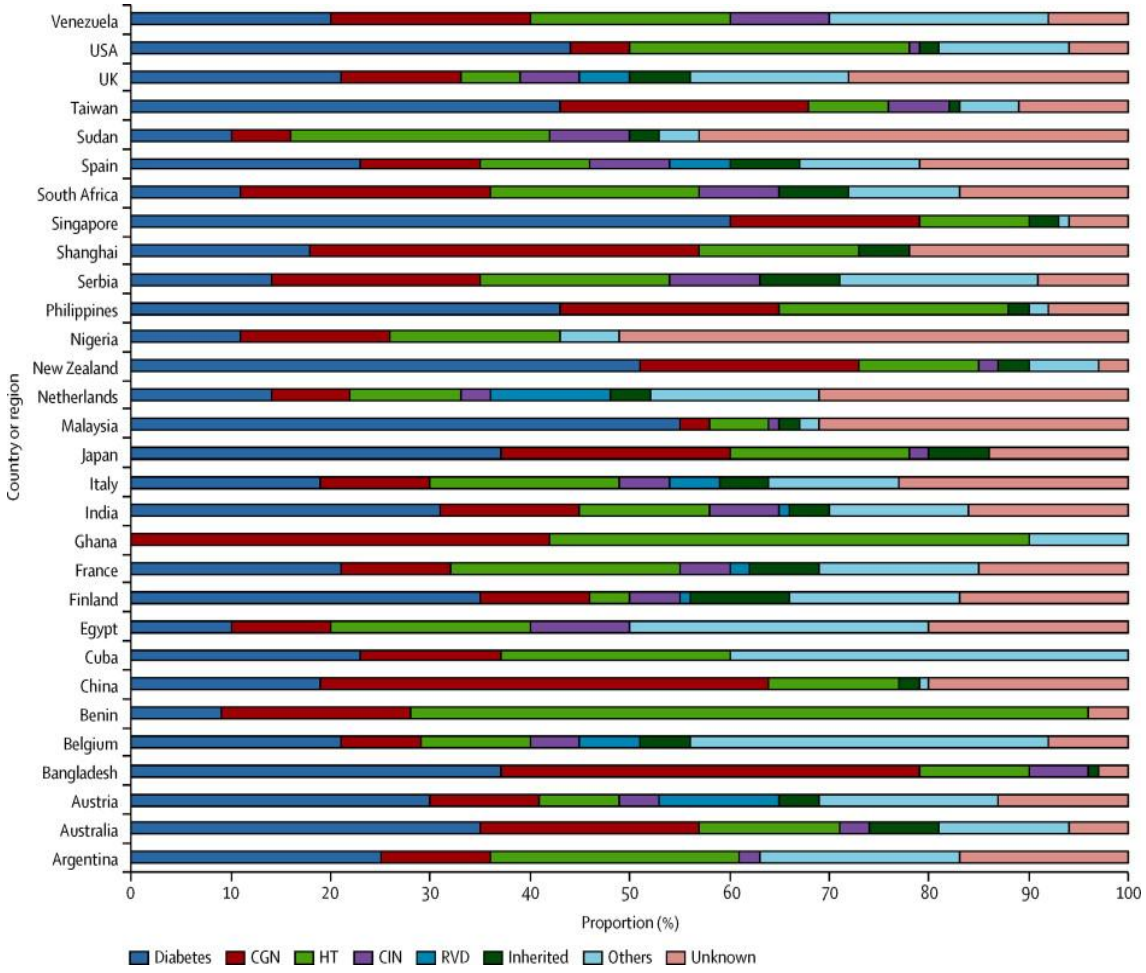


Figure 13: global causes of CKD. (CGN=chronic glomerulonephritis, HT=hypertensive nephrosclerosis, CIN=chronic interstitial nephritis, RVD=renovascular disease) **(Jha et al., 2013)**

Below are discussed the 2 major causes of CKD: diabetes and hypertension.

2.5.1. Diabetic nephropathy

About 25 - 40% of patients suffering from diabetes, either type I (T1D) or type II (T2D), will be diagnosed later with diabetic nephropathy (DN) **(Conserva et al., 2013)**.

The clinical history steps of a typical patient are **(Schena and Gesualdo, 2005)**:

1. Symptoms of hyperfiltration (elevated values of GFR) and occasional microalbuminuria, which may last approximately 5 years.
2. During the following ~20 years, microalbuminuria turns into progressively higher proteinuria, whereas GFR declines.
3. The patient undergoes renal insufficiency with severe proteinuria, which eventually evolves towards ESRD.

The pathophysiology of DN involves progressive thickening of the glomerular basement membrane, podocyte loss and mesangial proliferation in the glomerulus, and tubulointerstitial fibrosis characterized with uncontrolled deposition of extracellular matrix and low-grade inflammation **(Brosius, 2008, Lan, 2012, Conserva et al., 2013)**.

It is important to consider that hyperglycemia is a primary initiator of diabetic nephropathy. Very early, hyperglycemia leads to vasodilation and increased GFR because of increase of endothelial NO synthase (eNOS) expression in afferent arteries and glomerular capillaries **(Sugimoto et al., 1998)**. Progressively, glomerular distension causes endothelial dysfunction and hemodynamic alterations, loss of the glomerular basement membrane (GBM) electric charge and GBM thickening, decreased number of podocytes, foot process effacement and mesangial expansion as signs of the initial glomerular injury which eventually leads to glomerulosclerosis **(Lehmann and Schleicher, 2000, Wolf and Ziyadeh, 2007, Munusamy and MacMillan-Crow, 2009)**.

This glomerular damage causes tubular injury by different mechanisms. Proteinuria derived from GBM alterations activates tubular cells to produce pro-fibrotic mediators (TGF-β, angiotensin-II, etc.) and proinflammatory cytokines and promotes cell death. Moreover, microangiopathy results in reduced postglomerular blood flow to peritubular capillaries. The result is the activation of mechanisms responsible for tubular cell death, EMT, cell infiltration, tubule degeneration and interstitial fibrosis **(Remuzzi et al., 2006, Ziyadeh and Wolf, 2008)**. As a result, diabetic nephropathy is a disease affecting the whole nephron.

2.5.2. Hypertensive nephropathy

Hypertension causes a nephrosclerotic glomerulopathy characterized by (1) renal vasculopathy affecting preglomerular arteries and arterioles, endothelial dysfunction, wall thickening and fibrosis; (2) microvascular disease of the glomerular tuft capillaries; (3) diffuse glomerulosclerosis and, less often, focal and segmental glomerulosclerosis (FSGS), involving damage to the filtration barrier constituents (podocytes, mesangial cells and basement membranes); and (4) interstitial fibrosis

(Rosario and Wesson, 2006). Overall, arteriolar vasculopathy, vascular obstruction and decreased vascular density lead to decreased renal blood flow. However, GFR initially stays relatively stable. This is due to increased glomerular capillary pressure resulting from deficient or upwardly reset renal autoregulation and due to damage to the filtration barrier resulting in greater permeability. In the end, GFR decreases because of a progressive loss of surface area, mesangial hypertrophy and increasing glomerular and peritubular fibrosis. Moreover, basement membrane alterations induce albuminuria and protein hyperfiltration **(Lopez-Novoa et al., 2010)**.

2.6. Current detection and treatment of CKD

2.6.1. Detection

Current clinical detection of kidney damage is based on pathological persistent albuminuria (>30 mg/g creatinine), urine sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology, structural abnormalities detected by imaging or history of kidney transplantation. In addition kidney damage is also established based on the calculation of a decreased eGFR (<60 ml/min/1.73 m2). **(Otero et al., 2010, Stevens et al., 2010, Coresh et al., 2014, Parving et al., 2015)**. However, despite the importance of for example albuminuria in the clinical practice, this marker frequently falls short in the selection of individuals that would benefit most from timely therapeutic interventions **(Mischak et al., 2015, Miller et al., 2009)**. Furthermore, eGFR informs on the actual-, but not on future-renal function, that necessitates multiple measurements. Finally, a low eGFR (<60 ml/min/1.73 m2) is a late marker of disease where (irreversible) damage is already advanced.

A number of laboratories have tried to identify novel markers of CKD and its progression. These candidate markers include: 1) kidney injury molecule (KIM-1), a transmembrane protein increased in urine and plasma after kidney injury and associated with fibrosis, inflammation and acute tubular necrosis (ATN) in CKD patients **(Han et al., 2002, Hsu et al., 2009)**, 2) the neutrophil gelatinase-associated lipocalin (NGAL) a lipocalin iron-carrying protein which is expressed at tubular renal epithelial cells after tubular injury and increases in serum and/or in urine is a strong marker of CKD diagnosis and prognosis **(Mishra et al., 2004, German et al., 2008, Addo-Quaye et al., 2008, Llave et al., 2002)**, 3) fibroblast growth factor 23 (FGF-23) a phosphaturic protein secreted from bone osteocytes and has been shown to correlate with CKD progression **(Nakai et al., 2010, Isakova et al., 2011)** and, 4) monocyte chemoattractant protein 1 (MCP-1) an inflammatory chemokine which is upregulated in serum and urine in kidney diseases, including CKD **(Melgarejo et al., 2009, Wada et al., 2000, Thomson et al., 2011)**. Finally, a new approach to identify reliable biomarkers in the early stage of the development of CKD involves a panel of urinary peptides, which is shown to have better clinical value as a marker for CKD progression compared to albuminuria changes in the early stages of CKD **(Rosenfeld et al., 2008).**

2.6.2. Treatment

At the early stages of CKD the current treatment is focus on drugs that block CKD progression by controlling blood pressure. Two are now the most recognized:

1. Renin–angiotensin aldosterone system (RAAS) inhibition: RAAS inhibition has shown to be the most effective therapy at reducing proteinuria and slowing CKD progression in animals and humans **(Perico et al., 2008)**. Furthermore, in experimental models, RAAS inhibition has led to regression and repair of certain aspects of CKD, such as glomerular fibrosis **(Fogo, 2006, Remuzzi et al., 2006)**.

   RAAS inhibitors are considered as first in line therapy for patients with diabetic and non-diabetic kidney disease **(Brewster and Perazella, 2004)**. Clinical studies, trials and experience over the last several decades with RAAS blockers have demonstrated that RAAS inhibition using angiotensin converting enzyme inhibitors (ACEIs) and angiotensin AT-1 receptor antagonists (ARA) is the most effective single therapy to attenuate progression of CKD, despite being almost equally effective to other antihypertensive drugs at reducing blood pressure **(MacKinnon et al., 2006)**.

   The highly active octapeptide angiotensin II is produced by an initial cleavage of angiotensinogen by the enzyme renin into the decapeptide angiotensin I, which in turn is cleaved by the zinc metallopeptidase ACE and other carboxypeptidases. Angiotensin II has functional and structural effects on the vascular wall, heart, renal tissues, nervous system and many others **(Mire et al., 2005)**. ACEIs and ARA are the two families of compounds for RAAS inhibition **(Sica, 2005)**. Both drug types are clinically approved and commonly used for the treatment of hypertension as well as other cardiovascular disorders. Good pharmacological profiles with high efficacy and low toxicity characterize both families of compounds. ACEIs (e.g. enalapril, trandolapril, ramipril, lisinopril, benazepril and fosinopril) prevent angiotensin II from being produced by ACE, whereas ARA (e.g. losartan, valsartan, irbesartan, candesartan, eprosartan, olmesartan and telmisartan), which are selective for the AT-1 receptor prevent angiotensin II from binding and activating its receptor. RAS inhibitors are superior to other antihypertensive agents at reducing progression of CKD, proteinuria and mortality end-points not only due to blood pressure control, but also holding pressure-independent, beneficial actions on diseased kidneys **(Pugsley, 2005, Lewis, 2002)**.
2. Calcium channel antagonists: Calcium channel antagonists (CAs) are a family of compounds that inhibit L and T voltage dependent calcium channels. There are two categories of molecules: the dihydropyridine (nifedipine, nisoldipine, amlodipine, nicardipine, nitrendipine) and the non-dihydropyridine (verapamil, diltiazem) chemical structures. Each category acts differently because of lipophilicity differences. Non-dihydropyridines have both vascular and cardiac effects (vasodilation and negative inotropy, respectively), whereas dihydropyridines are quite selective for the vasculature **(Abernethy and Schwartz, 1999)**. CAs are widely

used for the treatment of cardiovascular diseases, including hypertension, whose main side effect is oedema of the lower extremities **(Weir et al., 2001, Nathan et al., 2005)**. CAs attenuate hypertension-related renal damage progression, when administered during incipient, non-proteinuric stages of disease, as all other antihypertensive agents **(Nathan et al., 2005).** In these cases, both dihydropyridines and non-dihydropyridines substantially reduce progression rate and proteinuria with non-dihydropyridines, to be mostly preferred, as they are more effective, in overtly proteinuric renal disease and renal dysfunction, especially in non-insulin dependent diabetic nephropathy **(Nathan et al., 2005, Nielsen and Flyvbjerg, 2000)**.

In general, both subclasses of CAs, help to the control of blood pressure, either alone or in combination with ACEIs or ARA, and exert beneficial effects on renal damage and progression of renal dysfunction **(Segura et al., 2005, Nielsen and Flyvbjerg, 2000)**. In addition, studies with CAs suggest that these molecules provide an additive renal protective effect in multidrug therapies (along with RAAS inhibitors), an effect independent from blood pressure reduction. This is because CAs acts on the renal vasculature by producing vasodilation preferentially at the afferent level, which results in an increased GFR **(Epstein, 1993)**.

2.7. The future needs in CKD

The main need in CKD is observed in the development of novel diagnostic and risk stratification tools and tools to personalize therapeutic approaches. To do this, accurate, sensitive, specific and non-invasive diagnostics tests should be designed to allow the identification of the etiology of CKD both early in the course of the disease and when patients seek medical care at a later stage. In addition, therapies that can slow-down or halt the progression CKD are currently lacking. Ideally therapies should be able to reverse established kidney damage **(Ortiz, 2015, Lopez-Novoa et al., 2010)**.

2.8. Urinary miRNAs as potential biomarkers of CKD

Since miRNAs are involved in so many (patho)physiological processes it is not a surprise that laboratories have been studying association of urinary miRNAs with CKD.

2.8.1 CKD, all cause

One of the first reports on urinary miRNAs in CKD described significantly increased levels of miR-638 in urine samples from patients with stages 3, 4 and 5 (ESRD) compared to samples from healthy volunteers **(Neal et al., 2011)**. MiR-638 has been shown to be associated with the maintenance of the epithelial cell phenotype **(Ma et al., 2014)**. In another report, differential urinary miRNA abundance was found to be associated to different CKD etiologies: reduced levels of urinary miR-15 were observed in diabetic glomerulosclerosis, increased levels of miR-17 in IgA nephropathy and increased miR-216a and miR-21 levels in hypertensive nephrosclerosis **(Szeto et al., 2012)**. More importantly, urinary miR-21 and miR-216a abundance was correlated with the rate of renal function decline **(Szeto et al., 2012)**. miR-21, in particular, is known to participate in the development of kidney fibrosis, a key mechanism leading to CKD **(Patel and Noureddine, 2012, Chau et al., 2012, Glowacki et al., 2013, Wang et al., 2012b)**. Reduced urinary abundance of miR-29a, miR-29b, miR-29c and miR-200a, miR-200b and miR-200c, have been observed in CKD patients originating from a variety of etiologies including diabetic nephropathy, focal segmental glomerulosclerosis, IgA nephropathy, membranous nephropathy and mesangial proliferative glomerulonephritis **(Lv et al., 2013b, Wang et al., 2011a, Oba et al., 2010, Qin et al., 2011, Tang et al., 2013)**. As miR-21, these miRNAs are known to be associated with the development of kidney fibrosis. MiR-29, known as an antifibrotic miRNA (see above section *Renal Fibrosis*, page 48) is also reported to control the expression of Tropomyosin 1 (TPM1), a protein involved in the epithelial to mesenchymal transition (EMT) mechanism, and collagen isoform COL2A1 **(Fang et al., 2013b, Wang et al., 2012a)**. The mir-8 family (miR-200a, miR-200c, miR-141, miR-429) is found to target ZEB1, ZEB2 and SIP1 known to be involved in the EMT process, in fibrosis and in maintenance of the cell phenotype **(Gregory et al., 2008, Xiong et al., 2012).**

2.8.2. Diabetic CKD

MiRNAs have also been shown to have an active role in diabetic nephropathy (DN), the main cause of CKD. Argyropoulos et al. studied urinary miRNA abundance in either normo-, micro- or macroalbuminuric type 1 diabetic (T1D) patients and in patients with overt diabetic nephropathy. They observed that urinary levels of miR-323b-5p, miR-429 and miR-17-5p were associated with persistent micro-albuminuria in patients with long standing T1D **(Argyropoulos et al., 2013)**. After predicting the targets of these miRNAs, it was hypothesized that miR-323b-5p may regulate Claudin-16, which is a vital component of the tight junction in the thick ascending limb of Henle in the tubular section of

the kidney **(Hou et al., 2007)** and that miR-17-5p targets fibronectin **(Shan et al., 2009)**, an extracellular matrix protein increased in DN **(Miller et al., 2014)**. In addition, miR-429 belongs to the same fibrosis-related mir-8 family mentioned above **(Padmashree and Swamy, 2013)**.

Another study analyzed the abundance of urinary miRNAs in urine of T1D patients with microalbuminuria as well **(Barutta et al., 2013)**. The authors observed decreased urinary levels of exosomal miR-155 and miR-424 and increased levels of miR-145 in T1D patients with microalbuminuria compared to T1D patients with normoalbuminuria or non-diabetic controls **(Barutta et al., 2013)**. Interestingly, *in vivo* and *in vitro* studies suggest that glomerular miR-145 overexpression might be triggered by hyperglycaemia, potentially stimulating mesangial cell hypertrophy and cytoskeleton remodeling, key early features of DN **(Wiggins et al., 2010, Cordes et al., 2009)**. MiR-424 was found to be involved in the regulation of angiogenesis **(Ghosh et al., 2010, Chamorro-Jorganes et al., 2011)** and miR-155 has been shown to regulate angiotensin 2 **(Martin et al., 2006)** and SMAD proteins related to fibrosis **(Louafi et al., 2010, Rai et al., 2010)**.

Besides glomerular and interstitial damage, DN is also characterized by vascular lesions. Liu et al. found that miR-126 abundance was increased in urine of type 2 diabetic (T2D) patients suffering from DN compared to diabetic patients without DN and healthy volunteers. MiR-126 is a miRNA highly enriched in endothelial cells and is a key player in maintaining endothelial homeostasis and vascular integrity. The authors therefore suggested that urinary miR-126 originates from endothelial cells-derived exosomes and propose that urinary miR-126 could serve as a biomarker to identify T2D patients with DN and monitor progression of the disease **(Liu et al., 2014)**, although this remains to be confirmed in independent studies.

2.8.3. Glomerulopathies

Primary glomerulopathies, such as minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous glomerulonephritis (MGN), immunoglobulin A nephropathy (IgA Nephropathy) and membranoproliferative glomerulonephritis (MPGN) are disorders affecting the glomerular structure and function **(Jiang et al., 2013, Glassock and Cohen, 1996)**.

IgAN is characterized by mesangioproliferative changes in glomeruli with deposition of Immunoglobulin A in the mesangium **(Szeto and Li, 2014)**. Research on miRNAs in urine of IgAN patients detected decreased abundance of miR-200a, miR-200b and miR-429 (mir-8 family) and correlated with disease severity and rate of progression. Decreased urinary levels of miR-429 were also found to be correlated to an increased rate of renal function decline **(Wang et al., 2010a)**. Furthermore, it has been shown that increased levels of miR-146a and miR-155 in urine and tissue are associated with reduced production of pro-inflammatory cytokines interleukin IL-1β, IL-6 and tumor necrosis factor TNFα, molecules involved in the pathogenesis of IgAN **(Wang et al., 2011b, Faraoni et al., 2009, Qu et al., 2014)**.

Wang et al. demonstrated that the levels of urinary miR-10a and miR-30d were elevated in patients suffering from FSGS compared to healthy donors **(Wang et al., 2012c)**. MiR-10a and miR-30d are reported as kidney specific miRNAs serving a protective role against kidney tissue injuries by targeting IL-12/IL-23p40 **(Xue et al., 2011)**, the pro-apoptotic protein Bim **(Chu et al., 2014)**, apoptotic caspase CASP3 **(Li et al., 2012)** and tumor suppressor gene p53 **(Kumar et al., 2011)**.

In another report, urinary miR-155, miR-196a, miR-30a-5p, and miR-490 levels have been shown to increase in FSGS patients compared to healthy controls, and moreover miR-196a, miR-30a-5p, and miR-490 levels significantly decreased in FSGS patients under treatment with steroids, a common immunosuppressive therapy employed in FSGS patients leading to remission **(Ren et al., 2013)**, suggesting a potentially predictive use for the response on steroid therapy **(Zhang et al., 2014)**.

2.9. Conclusion

CKD is an ongoing worldwide burden in public health. It affects the quality of life of millions of people, placing CKD as the fastest growing causes of premature death worldwide. The main issues remain the heterogeneity of the disease and the lack of tools for efficient diagnosis and prognosis. miRNAs have been shown to play a key role in different pathological conditions, including CKD, and could serve as biomarkers and/or therapeutic targets. Urinary miRNAs may also serve as non – invasive biomarkers, a tool needed for accurate and fast diagnosis and prognosis of CKD, to replace kidney biopsies.

3. OBSTRUCTIVE NEPHROPATHY AND miRNAs

3.1 Introduction

In contrast with CKD in adults, which for the greater part originates from T2D and hypertension **(Zhu et al., 2007),** the most common cause of CKD in children are congenital anomalies of the kidney and the urinary tract (CAKUT) counting for more than half of the incidence of CKD in children. Furthermore congenital obstructive nephropathy (CON) is the main cause of ESRD in children **(Thompson et al., 2015)**.

3.2 Ureteropelvic junction (UPJ) obstruction

The most frequent cause of congenital urinary tract obstruction is ureteropelvic junction (UPJ) obstruction with an estimated incidence of 1 in 1000–1500 **(Chang et al., 2004)**. It is caused by a stenosis of the ureter at the connection point with the pelvis which prevents the normal flow of the urine to the bladder, resulting in severe cases of obstruction in hydronephrosis (enlargement of the kidney because of urine accumulation **(National Kidney, 2002)** (Figure 14)**.**

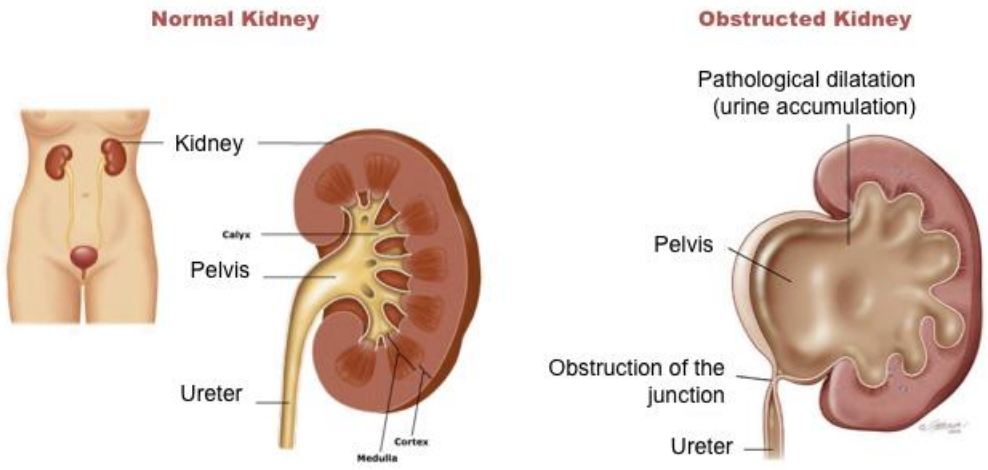


Figure 14: A stenosis of the ureter at the connection point with the pelvis results in obstructive nephropathy.

3.2.1 Histological and functional changes in UPJ obstruction

The histological alterations of the kidney varies greatly in UPJ obstruction ranging from subtle changes such as modified proximal or tubular size, chronic tubulointerstitial injury, glomerulosclerosis, fibrosis, aberration of nephron development and in severe cases (less than 1%) renal dysplasia **(German et al., 2008)**. Altogether, reduction of nephrogenesis associated with glomerular and tubular apoptosis lead to a significant reduction in the number of functional nephrons **(Addo-Quaye et al., 2008, Llave et al., 2002)**. All these changes affect the function of the kidney by reducing the renal blood flow, the glomerular filtration rate and the reabsorption of water and solutes from the tubules (e.g. sodium reabsorption, solute-free water) **(Klein et al., 2011)**.

A list of histological lesions modifications and functional modifications in obstruction is summarized in figure 15.

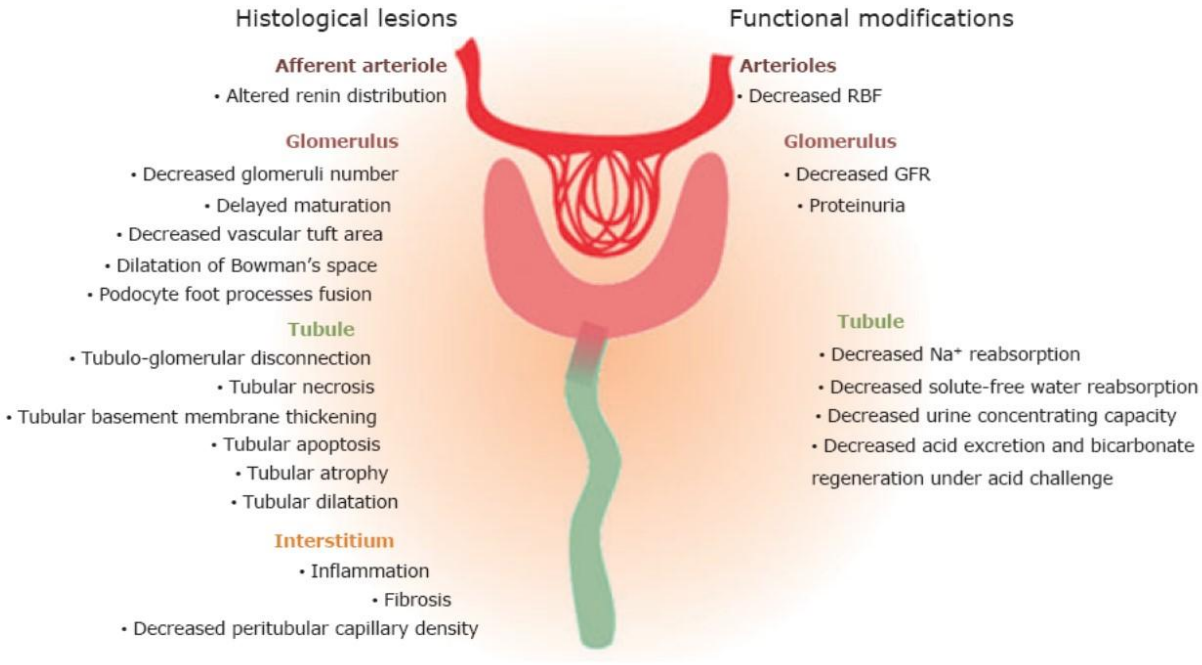


Figure 15: UPJ obstruction: histological modifications and functional modifications in obstruction **(Klein et al., 2011**)

3.2.2 Diagnosis and management of UPJ obstruction

The gold standard in diagnosis of UPJ obstruction is by prenatal ultrasonography with subsequent evaluation in the postnatal period **(Chevalier, 2015b)**. However, this method is not sensitive enough to accurately estimate renal function and functioning nephron number **(Waikar et al., 2009)**. Approximately 5% of pregnancies are diagnosed with fetal hydronephrosis, but after birth most infants do not show signs of obstruction, while around 1.5% of the initial screening population may need long term health care **(Parving et al., 2015, Mischak et al., 2015)**. This uncertainty on the severity of the status of the obstruction has led to a debate among the nephrologists and pediatricians about the necessity and the time to perform corrective surgery, pyeloplasty. On one hand, surgery, and especially surgery on infants and babies is not innocuous. On the other hand, a "wait and see" approach in order to allow the full development and stability of the kidney bears the risk of waiting too long, and to damage parts of the kidney. This has led to an urgent need for the development of biomarkers to assess the severity of UPJ obstruction and to help the clinicians to decide if and when pyeloplasty is required **(Chevalier, 2015b, Chertin et al., 2006)**.

Furthermore, it is becoming increasingly clear that patients with congenital urinary tract obstruction must be followed into adulthood, as the lesions can progress over the entire life and the optimal biomarkers need to be age-specific to follow all stages of a patient's development from birth to childhood and adulthood **(Zhu et al., 2007, Chevalier, 2016).** To this direction, a panel of 53 urinary polypeptides has been proposed as a marker to estimate the severity of the obstruction and the immediate need for pyeloplasty or not **(Varadhachary et al., 2011, Mueller et al., 2011)**. Finally, a label-free quantitative urinary proteomics study in individuals with UPJ obstruction and in mouse model of obstructive nephropathy suggested that the arginine metabolism is disturbed in obstructive nephropathy **(Meiri et al., 2012)**.

3.2.3 Animal models for better understanding of the pathophysiology of UPJ obstruction

Due to the fact that limited human kidney samples are available, almost all observations on the pathophysiology of UPJ obstruction are based on animal models, which potentially limit the transferability of the observations in the clinical context. As a consequence, the pathophysiological mechanisms of UPJ obstruction remain incompletely understood, even though the modifications of the renal lesions in UPJ obstruction are known (tubular proliferation/apoptosis, renin-angiotensin system activation, inflammation, and fibrosis) **(Klein et al., 2011, Chevalier, 2015b, Chevalier et al., 2014, Meiri et al., 2012)**.

One factor for the lack of understanding of the mechanisms is the difference in the development of the kidney in human and mice and rats. Humans are born with fully developed urinary system (kidney, urinary tract etc.) while in mice and rats it is finalized after birth until 18th postnatal day **(Nishimura et**

al., 1999). To study the outcomes of obstruction, surgical animal models of unilateral ureteral obstruction (UUO) have been developed in adult rodents and mice. These models are excellent to study tubulointerstitial fibrosis in severe obstruction, but fail to recapitulate what happens during the development of the kidney in which obstruction can interfere with kidney morphogenesis, growth and maturation **(Ong et al., 2002)**.

In order to better mimic UPJ in the developing kidney, two rodent models of UUO have been developed in neonatal mice or rats. The first is complete UUO, achieved by a simple suture ligation of the ureter to mimic severe UPJ obstruction, progressing fast to hydronephrosis and loss of renal parenchyma (65%). Complete UUO in neonatal mice impairs nephron maturation and tubular oxidative stress, activates intrarenal renin-angiotensin system, promotes the production of TGF-β and fibrosis and as the injury progresses macrophages and fibroblasts are also entering the game **(Zhu et al., 2007, Chevalier, 2016)**. The second UUO model, which mimics better the conditions during human kidney development with obstruction, is the partial neonatal UUO. A stainless steel wire of known diameter is placed in parallel to the ureter, followed by ligature of both the ureter and the stainless steel wire. Once the wire is withdrawn the result is a well calibrated partial obstruction. This technique offers reproducibility and by choosing the diameter of the wire, control of the severity and the duration of the obstruction **(Hafner et al., 2010)**. Experiments also revealed that individual nephron responses to partial neonatal UUO in the developing kidney are heterogeneous, with some nephrons showing reduced tubular fluid flow, while others maintain their normal function **(Kulkarni et al., 1971)**.

3.3 miRNAs in Obstructive nephropathy

Few reports are available on miRNAs and their role in obstructive nephropathy and all studies used the complete adult UUO model. Most reports focus on the role miRNAs have to control the TGF-β/Smad pathway responsible for kidney fibrosis **(Chevalier, 2016)**. In particular, Li *et al.* observed that miR-483-5p and miR-483-3p may be involved in renal fibrosis development in the adult mouse model of complete UUO by promoting the expression of their host gene *Igf2* and other cytokines **(Li et al., 2015b)**. Two reports support the therapeutic potential of the use of miRNAs against renal fibrosis using the complete adult UUO model. In both reports, miRNAs were injected in the tail vein during UUO (mimic of miR-34c and polyethylenimine nanoparticles with miR-146 respectively) and in both occasions renal fibrosis attenuation was observed **(Morizane et al., 2014, Morishita et al., 2015)**. Administration of mimic-34c led to decrease of the kidney fibrosis area, inhibition of EMT and the expression of fibrotic molecules including connective tissue growth factor, α-SMA, collagen type 1, collagen type 3 and fibronectin **(Morizane et al., 2014)**, while administration of miR-146 contained

renal fibrosis by inhibiting the transforming growth factor beta 1–Smad and tumor necrosis factor receptor-associated factor 6–nuclear factor kappa B signaling pathways **(Morishita et al., 2015)**.

There are also reports that correlate miRNAs with development of CAKUT, not necessarily caused by obstructive disease. For example, Medrano *et al.* showed that miR-143/145 deficient mice developed hydronephrosis rapidly after birth (within 2 weeks) due to abnormal ureter peristalsis but without any obvious signs of obstruction **(Medrano et al., 2014)**. Interestingly, one of the most important reports on the key role the miRNAs have in kidney development that could lead to CAKUT was by Bartram *el al.* In this report, knocking down Dicer (the protein responsible to produce the mature miRNA during miRNA-biogenesis) in embryotic mice resulted in alterations in morphology of the kidneys, the ureters and reduction in tubular branching **(Bartram et al., 2013)**. Further reports from the same team on Dicer and Drosha/Dgcr8 complex revealed that any obstacle in the biogenesis process of miRNAs can lead to poor development of renal structure and function (Bartram et al., 2016).

Finally, since obstructive nephropathy is characterized by fibrosis, miR-21 could not be absent from studies related to UUO. The activation of the TGF-β/Smad signaling promotes the expression of miR-21 in UUO mouse model and blocking miR-21 in a UUO model reduced the deposition of collagen, fibronectine, SMA-a and PAI-1 and macrophage infiltration, molecules that are up-regulated in fibrosis, showing, in total, not only the key role of miR-21 in fibrosis and UUO but also the therapeutic value of the inhibition of this miRNA **(Zarjou et al., 2011)**.

3.4 Conclusion

The main risk factor leading to pediatric kidney disease is obstructive nephropathy due to developmental disease. The challenge of research is to enrich the limited knowledge and understanding on the pathological mechanisms responsible for obstructive nephropathy true the use of UPJ obstruction models. To this direction are focused the experiments on the animal model closest to human pathology, the partial UUO mouse model. A second challenge deriving from the use of the UUO animal model is the transferability of the results to human pathology. Finally, there is a strong need for non-invasive biomarkers which can diagnose and predict with high specificity and sensibility the severity of the condition of the kidney in an infant or a child and guide the nephrologist to the correct decision for monitoring and treatment.

4. OBJECTIVE OF THE THESIS

miRNAs are now recognized as key players in the regulation of proteins and any abnormality in their function is a cause for pathway instability, leading to pathological conditions. Numerous reports from a variety of pathologies provide new data about miRNAs function, their targets, their potential as biomarkers and possible ways to control miRNAs' expression for potential therapeutic purpose.

A number of reports connect miRNAs with pathological conditions in the kidney and point to the use of miRNAs as biomarkers for diagnosis and prognosis of kidney disease in blood, serum, tissue and urine samples. In this thesis, we researched:

1) a possible role of the miRNAs in the progression of adult CKD, a disease representing a global burden with the tendency to rise worldwide. Progression of CKD is still very hard to detect non-invasively with the currently used clinical tools (eGFR and albuminuria). In our work we attempted to identify alterations of the level of the miRNAs in human urine samples and characterize a panel of miRNAs as biomarkers for non-invasive progression of CKD.

2) the presence of urinary miRNAs associated to obstructive nephropathy, a frequently encountered disease in children that can lead, in severe cases, to early ESRD. In this study we used a comprehensive system biology study in which we combined mi- and mRNA data from human and animal obstructive nephropathy to obtain information on possible mechanisms involved in obstructive nephropathy.

5. ASSOCIATION OF URINARY MIR-145 TO PROGRESSION OF CHRONIC KIDNEY DISEASE.

5.1. The context of this study: The iMODE – CKD project.

iMODE-CKD (*Clinical and system –omics for the **i**dentification of the **MO**lecular **DE**terminants of established **C**hronic **K**idney **D**isease)* is a European Project which integrates multi-disciplinary expertise in proteomics, metabolomics, transcriptomics, bioinformatics, pathology, and clinical science from leading academic and industrial investigators, establishing a unique training platform on biomarker research and Systems Biology. The main goals of the iMODE-CKD are to improve the quality of life of patients with chronic kidney disease (CKD) and diminish the severe health and economic burden imposed by this disease, by providing better diagnostic and prognostic means. In order to do so, we aimed to identify and validate molecules involved in progression of renal complications trough clinical –omics in established CKD and to understand the molecular determinants of established CKD through integrative Systems Biology.

As part of iMODE - CKD this study focused on miRNA modifications in urine samples of established CKD patients and correlate their expression profiles to disease progression. Global miRNA was detected by Next Generation Sequencing (NGS). Differentially expressed miRNAs in relation to CKD progression that survive multiple testing were validated using Applied Biosystems low-density miRNAs arrays in a new set of patients. Targets of validated differentially expressed miRNAs were selected using pathway analysis and correlated to proteomics changes. The most significant miRNA-mRNA/protein correlations were validated in renal HK2 cells by modification of expression of selected miRNA (antagomers) and study of effects on target mRNAs.

5.2 ABSTRACT

Chronic kidney disease (CKD) has become one of the major non-communicable diseases and its management implies early detection and identification of individuals that display progressive disease. Body fluid miRNAs have been increasingly found to be associated to (kidney) disease and might serve as markers of CKD progression as well. In this study, using Next Generation Sequencing, we analyzed urinary miRNA modifications in urine samples of 70 patients with established CKD, 35 non-progressors (≤2% reduction and ≤4.5% increase of estimated glomerular filtration rate (eGFR) slope per year) and 35 progressors (≥3.3% reduction of eGFR slope per year). We correlated the miRNA expression profiles to disease progression. This lead to the identification of 24 urinary miRNAs that were significantly associated to CKD progression. The combination of the 24 miRNAs with 142 proteins, found statistically different in the same cohort of samples, led to the selection of 4 miRNAs for validation in a new cohort of 52 patients (19 non-progressors and 33 progressors). Among these four, we validated the increased abundance of hsa-miR-145-5p in urine of patients with progressive CKD using Taqman based technologies. Urinary abundance of hsa-miR-145-5p was significantly inversely correlated to eGFR decline ($r=-0.5$, $p=0.02$) and showed a possible predictive marker value (AUC 0.733). Proteome analysis of HK2 kidney cells with artificially reduced expression of hsa-miR-145-5p predicted an association of this miRNA to necrotic processes. In conclusion we have identified hsa-miR-145-5p as potential urinary miRNA marker of CKD progression that could be linked via its targets to CKD progression.

5.3 INTRODUCTION

Chronic kidney disease (CKD) is a general term for kidney failure classified into 5 stages defined by the decline in the glomerular filtration rate (GFR) **(Levin and Stevens, 2014).** It is characterized by the progressive loss of kidney function and is considered as a global major health burden and is associated with high-cost of treatment **(Jha et al., 2013)**. The Global Burden of Disease study data (GBD2013) **(De Nicola and Minutolo, 2016)** showed that CKD was the fastest growing non-communicable cause of death. Multiple factors contribute to the incidence and prevalence of the disease, including age, diabetes, hypertension, obesity, smoking and autoimmune diseases **(Jha et al., 2013)**. CKD is most frequent in elderly with around two thirds of persons aged 80, or more, having CKD **(Stevens et al., 2010).** Progression of CKD ultimately leads to end-stage renal disease (ESRD) which occurs when the kidneys functions are impaired to a very large degree and thus cannot support everyday needs of the organism. When a patient reaches ESRD, he or she has to undergo kidney transplantation or dialysis to survive.

The main issues in CKD management are 1) the early detection of disease in patients at risk (*i.e.* in individuals with diabetes, hypertension, etc) and 2) the identification of patients with already established CKD that will (rapidly) progress towards ESRD **(Ecder, 2013, Lutz et al., 2014)**. Identification of individuals with CKD is currently depending on a reduced estimated GFR (eGFR) **(Coresh et al., 2014)** and/or increase of urinary albumin excretion **(Parving et al., 2015)**. However, even if routinely used in the clinic, albuminuria often falls short to identify individuals with progressive CKD and several eGFR measurements are necessary to firmly establish disease progression **(Pontillo et al., 2016)**. To tackle this issue, several putative urine and plasma biomarker candidates for the estimation of CKD progression have emerged. The most classical example is proteinuria. However, proteinuria may lack appropriate specificity, as its variability (especially in the case of response to treatment) indicates that it may be an appropriate surrogate for progression only in some kidney diseases **(Levey et al., 2009)**. Other prominent examples of biomarkers include: neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 (KIM-1), liver-type fatty acid binding protein, fibroblast growth factors or tumour necrosis factor receptor 1 and 2 **(Fassett et al., 2011, Looker et al., 2015, Rebholz et al., 2015, Krolewski et al., 2014, Addo-Quaye et al., 2008, Devarajan, 2010, Hojs et al., 2015, Bhavsar et al., 2012)**. The clinical utility of these putative predictive biomarkers is currently evaluated and, as in the case of KIM-1 **(Bhavsar et al., 2012)**, the obtained results seem inconclusive, questioning their added value for the prediction of CKD progression. Therefore the field can benefit from the development of more sensitive and reliable biomarkers of CKD progression.

MicroRNAs (miRNAs) are small non-coding RNAs (20 - 24 nt) that regulate gene expression by blocking the translation of mRNAs into proteins and are involved in multiple molecular pathways and pathologies. MiRNAs are now considered promising molecules for biomarkers and/or targeted

therapy of multiple diseases **(Hammond, 2015, Almeida et al., 2011, van Rooij and Kauppinen, 2014, Esteller, 2011)**. Significant variations in urinary miRNA abundance have been associated to different CKD etiologies including diabetic glomerulosclerosis (decrease of miR-15), IgA nephropathy (increase miR-17) and hypertensive nephrosclerosis (increase of miR-21 and miR-216a) **(Szeto et al., 2012).** A number of specific miRNAs, known to be associated to renal fibrosis (a hallmark of CKD), including miR-192, miR-29a, miR-29b, miR-29c and miR-200a, miR-200b and miR-200c, displayed reduced urinary abundance in CKD **(Lv et al., 2013b, Szeto et al., 2012)**. In contrast, miR-21 is considered as a pro-fibrotic miRNA with increased urinary abundance in renal fibrosis **(Zununi Vahed et al., 2016).**

These observations suggest that studying urinary miRNAs in CKD progression might help to understand the development of CKD progression and/or provide markers for prognosis. Here we studied urinary miRNAs in a total of 122 patients with established CKD using Next Generation Sequencing (NGS) and TaqMan Low Density Arrays (TLDA) in combination with proteomics analysis to identify urinary miR-145 as potentially being associated to CKD progression. Proteome analysis from human renal HK2 cells treated with an agent blocking the action of miR-145 (antagomir) combined with pathway analysis revealed the association of this miRNA with necrotic processes.

5.4 MATERIALS AND METHODS

Study design and population:

Discovery set

A matched retrospective cohort study was conducted. The source population consisted of 491 patients aged from 17 years to 89 years old attending the Nephrology Outpatient Clinic at Ghent University Hospital, Ghent, Belgium (the "Ghent cohort"). Patients were recruited during their referrals at the Clinic. Patients with renal transplantation or receiving renal replacement therapy (hemo- or peritoneal dialysis) were excluded. Samples were collected in the period of 17/01/2011 to 07/07/2015. Sample collection was performed in accordance to local ethics requirements and the study was approved by the local ethics committee. All individuals gave written informed consent under Institutional Review Board approved protocols. Second morning mid-stream urine samples were collected. To remove cell debris, urine samples were centrifuged at 1,000xg for 10 min at 4˚C. To reduce freeze-thaw cycles, samples were aliquoted in 500 µL and stored at -20˚C. In cases protein precipitation occurred, prior to the protein concentration measurement with Bradford method, 10 to 20 µL of 1M ammonia solution (pH 10) was added to dissolve the precipitate.

The following clinical and laboratory measurements were collected for all participants: eGFR and CKD-stage, follow-up serum creatinine measurements, baseline and last follow-up date, number of time-points (referrals), annual % changes in eGFR slope and use of medication. Estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation **(Florkowski and Chew-Harris, 2011)**.

Of the original study population, 183 patients were excluded from the analyses as they displayed tubulointerstitial diseases, familial/hereditary nephropathies, and/or malignancies. Additional 44 patients were excluded as they did not have sufficient follow-up data, including at least 3 serum creatinine measurements and 2 years of clinical follow-up.

For the purpose of the study, progressors were defined as CKD patients with ≥3.3% reduction of eGFR slope per year (according to **(Merchant et al., 2009)**), while non-progressors were defined as CKD subjects with ≤2% reduction and ≤4.5% increase of eGFR slope per year.

Progressors were matched to non-progressors for multiple variables as sex, age, body mass index, pulse, systolic blood pressure, diastolic blood pressure, presence of diabetes, baseline serum creatinine and baseline eGFR leading to the selection of 70 patients including 35 non-progressors and 35 progressors as the final selection (**Table 1**). Statistical difference in the use of drugs between progressors and non-progressors was observed. Significantly more progressor patients were receiving the following drugs: angiotensin-converting-enzyme inhibitors and angiotensin II receptor blockers (ACEi and ARB; p=0.04), statins (p=0.01) and vitamin D (p=0.03). As expected, progressors showed a significantly higher average last follow-up creatinine concentrations and lower average last follow-

up eGFR (**Table 1**). A detailed description of the study population is presented in **Supplementary Table 1A**.

Table 1: Comparison of clinical data for CKD progressors compared to non-progressors of the discovery set. The patients are matched for gender, age, BMI, pulse, systolic blood pressure, diastolic blood pressure, presence of diabetes, baseline serum creatinine, and baseline eGFR. The patients were not matched for drug use. * denotes significant difference ($p \leq 0.05$).

Variable		All samples n=70	Non-progressors n=35	Progressors n=35	p-value Mann-Whitney
% change in eGFR/year (mean ± SD)		-6.7±9.1	0.3±1.5	-13.7±7.9	0.0001*
Gender [F/M]		23/47	11/24	12/23	0.81
Age [years]		65±15	63±15	67±15	0.10
Body Mass Index [BMI]		29±5	29±5	30±5	0.25
Systolic blood pressure [mmHg]		138±18	133±18	144±18	0.02*
Diastolic blood pressure [mmHg]		79±9	80±7	78±10	0.14
Pulse		68±12	67±11	69±13	0.56
Diabetes [Y/N]		25/45	11/24	14/21	0.47
Baseline Serum creatinine [mg/dl]		1.6±0.5	1.6±0.5	1.7±0.6	0.32
Baseline eGFR [ml/min/1.72]		46±19	49±19	42±18	0.10
Last follow-up serum creatinine [mg/dl]		2.1±1.2	1.5±0.5	2.6±1.5	0.0002*
Last follow-up eGFR [ml/min/1.72]		39±20	49±19	28±15	0.0001*
Drug use	ACEi and ARB [Y/N]	60/10	27/8	33/2	0.04*
	Statins [Y/N]	53/17	22/13	31/4	0.01*
	Anticoagulants [Y/N]	10/60	3/32	7/28	0.17
	Vitamin D [Y/N]	27/43	9/26	18/17	0.03*

Validation set

The validation cohort was comprised of patients from the Ghent cohort (see discovery) and the CKD-BIO cohort. The CKD-BIO cohort consists of 155 patients with CKD attending various clinical centers across the Europe (Belgrade, Leipzig, Reggio Calabria, Wroclaw, Madrid, Prague, Napoli, Nicosia, Tirana, Timisoara, Rijeka and Skopje). Sample collection began at 28/03/2012 and is still

ongoing. All sample collection procedures were performed in accordance to local ethics requirements and the studies were approved by local ethics committees.

To obtain the largest number of samples for validation the follow-up criteria were relaxed compared to the selection of the discovery cohort. Patients were included if at least 2 serum creatinine measurements were available for a minimum follow-up of one year. Moreover, patients reaching hemodialysis during follow-up period were also considered as progressors. This lead to the selection of 13 non-progressors and 11 progressors from the Ghent- and 6 non-progressors and 22 progressors from the CKD-BIO cohort yielding a total of 19 non-progressors and 33 progressors.

The following clinical and laboratory measurements were collected for all validation set participants: eGFR measurements by CKD-EPI equation, CKD stage by CKD-EPI equation, follow-up serum creatinine measurements, baseline and last follow-up date, number of time-points (referrals), annual % changes in eGFR slope and use of medication (**Supplementary Table 1B**). The cohort was not matched for any factors prior to the analysis, as in the case of discovery set. Nevertheless, no significant difference in gender distribution, age, diabetes, baseline serum creatinine, baseline eGFR or use of medicaments was observed (**Table 2**). Significant difference between CKD progressors and non-progressors was observed in the case of last follow-up serum creatinine and last follow-up eGFR.

Table 2: Comparison of clinical data for CKD progressors compared to non-progressors of the validation set. The patients show no statistical difference in case of gender, age, presence of diabetes, baseline serum creatinine, baseline eGFR values and use of medicaments. * denotes significant difference ($p \leq 0.05$)

Variable		**All samples n = 52**	**Non-progressors n = 19**	**Progressors n = 33**	**p-value Mann-Whitney**
% change in eGFR/year (mean ± SD)		-13.8±21.7	2.1±3.3	-23.6±22.4	0.0001*
Gender [F/M]		16/36	6/13	10/23	0.93
Age [years]		58±17	57±16	58±18	0.85
Diabetes [Y/N]		11/41	4/15	7/26	0.98
Baseline Serum creatinine [mg/dl]		1.70±0.80	1.38±0.43	1.89±0.91	0.09
Baseline eGFR [ml/min/1.72]		50.9±23.7	58.1±21.0	46.9±24.5	0.09
Last follow-up serum creatinine [mg/dl]		2.1±1.4	1.4±0.5	2.6±1.6	0.00024*
Last follow-up eGFR [ml/min/1.72]		43.6±23.7	58.0±21.8	34.8±20.5	0.00065*
Drug use	ACEi and ARB [Y/N]	36/16	12/7	24/9	0.49
	Statins [Y/N]	21/31	6/13	15/18	0.33
	Anticoagulants [Y/N]	3/35	1/18	4/29	0.38
	Vitamin D [Y/N]	14/38	5/14	9/24	0.94

RNA extraction (discovery and validation cohort)

Total RNA from the urine samples was extracted using Illumina's MasterPure RNA extraction kit according to manufacturer's guidelines. In detail, 500 μl of lysis solution (provided by Illumina's extraction kit) was added to the 500 μl of urine sample together with 3.5 μl of 50 μg/μl Proteinase K (Thermo Fisher Scientific) followed by incubation at 65^{o} C for 15 min. Next, 585 μl of MPC Protein Precipitation Reagent (provided by Illumina's extraction kit) were added, mixed, followed by centrifugation at 4°C for 10 minutes at ≥10,000 x g to pellet the debris. The supernatant was transferred to 15 ml falcon tubes and the pellet was discarded. This step was repeated two times. In the recovered supernatant 1700 μl of isopropanol was added followed by multiple inversions of the tube and by centrifugation at 4°C for 30 minutes at ≥4,000 x g in a microcentrifuge to pellet the nucleic acids. Then, the isopropanol was poured off and the total nucleic acid pellet was washed with 200 - 250 μl of 70% ethanol. Finally, the total nucleic acids were resuspended in 30μl of H_2O and stored in -20°C.

Next Generation Sequencing and data analysis (discovery cohort)

Library preparation and sequencing

The RNA quality and RNA concentration of each sample was performed using a Fragment Analyzer (Advanced Analytical Technologies). The sequencing library was built using the Illumina® Truseq small RNA Sample Prep Kit according to kit protocol "Truseq small RNA Sample Preparation" (15004197bRev. D). The obtained small RNA library of fragment length of approximately 150 bps was sequenced using the Illumina HiSeq 2500 platform.

Data processing

Clean sequenced reads were obtained by removing adapter sequences using Trimmomatic v0.30. Reads and were aligned against the human reference sequence GRCh37.75 using TopHat v.1.4.0 with Bowtie v.0.12.7. Feature counting of miRNAs was performed using HTSeq v.0.6.1p1 tools providing miRNA annotation in GTF format adapted from Ensembl *Homo sapiens* annotation v.75. The obtained miRNA expression data was used as the source file for downstream statistical analysis to determine differential expression of miRNAs using the DESeq package v.1.10.1 from the R statistical computing software.

TaqMan low-density microRNAs arrays and statistical analysis (validation cohort)

For the validation phase, the Custom TaqMan® Array MicroRNA Cards of Applied Biosystems (Publication Part Number 4478705) were used according to manufacturer's protocol. In brief, total RNA was extracted as mentioned above with Illumina's MasterPure RNA extraction kit. Next, by using 3μl of total RNA, a reverse transcription was performed with the custom RT Primer

Pool (Applied BioSystems, 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and pause at 4°C in a ThermoCycler). Next, 2.5µl of the RT product was used for the preamplification reaction with TaqMan® PreAmp Master Mix and Custom PreAmp Primer Pool (Applied BioSystems, 10 min at 95°C, 2 min at 55°C, 2 min at 72°C, 12 cycles of 15 sec at 95°C followed by 4 min at 60°C, 10 min at 99.9°C and finally, pause at 4°C in the Thermocycler). The preamplified product was diluted 1/20 with the PCR Master Mix (Applied BioSystems) and nuclease-free water and applied to Custom TaqMan® Array MicroRNA Cards. Analysis was performed using an Applied Biosystems 7900HT/7900HT Fast Real-Time PCR System with default settings. The analysis of the results was performed using Thermo Fischer Cloud 2.0.

Urine sample preparation for LC-MS/MS analysis (discovery cohort)

Five hundred µL of urine samples were processed following the FASP protocol, as described previously **(Filip et al., 2015)** with minor modifications. Specifically, if protein concentration was below the limit of detection for Bradford assay (≤0.2 µg/µL), 0.25 µg of trypsin per sample was added. Additionally, after peptide digestion, the concentration of peptide mixture in the sample was estimated using NanoDrop 2000 spectrophotometer (Thermo Scientific). The peptide mixture was lyophilized and stored at -20°C before reconstituted and loaded onto the LC-MS/MS column.

LC-MS/MS analysis

Eight µg of protein digest were loaded onto a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK). After loading onto a Dionex 0.1×20 mm 5 µm C18 nano trap column at a flow rate of 5 µl/min in 0.1% formic acid and 2% acetonitrile (ratio 98:2), samples were applied onto an Acclaim PepMap C18 nano column 75 µm×50 cm, 2 µm 100 Å at a flow rate of 0.3 µl/min. The trap and nano flow column were maintained at 35°C. The samples were eluted with a gradient of solvent A: 0.1% formic acid versus solvent B: 80% acetonitrile starting at 1% B for 5 min rising to 5% B at 10 min then to 25% B at 360 min and 65%B at 480 min.

The eluent was ionized using a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). Ionization voltage was 2.6 kV and the capillary temperature was 200°C. The mass spectrometer was operated in MS/MS mode scanning from 380 to 2000 amu. The top 20 multiply charged ions were selected from each scan for MS/MS analysis using HCD at 40% collision energy. The resolution in MS1 was 60,000 and 7,500 at m/z for HCD in MS2.

Protein identification and data processing

Protein identification and data processing was performed as previously described in **(Filip et al., 2015)**, with minor modifications. Specifically after merging of obtained sequences from all 70 analyzed samples and application of "Occam-Razor" rule for assigning of peptides to proteins, we considered for further differential expression and pathway analyses only peptides reported in more than 60% of the samples in at least one of the compared groups (22 out of 35 samples in at least one group).

Protein peak area was calculated based on the average peak area of top three most abundant peptides for a given protein. Subsequently, protein peak areas were normalized based on parts per million normalization followed by urine creatinine normalization, as follows:

$$PPM\ protein\ peak\ area = \frac{Average\ protein\ area\ based\ on\ top\ 3\ peptides}{Total\ peak\ area\ in\ the\ sample} * 10\text{^}6$$

$$Normalized\ protein\ peak\ area = \frac{PPM\ proein\ peak\ area}{urine\ creatinine\ concentration[\frac{mg}{dL}]}$$

Cell models and antagomir treatment

Human HK-2 cells were grown in a DMEM/F-12 Nut Mix medium supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO, Grand Island, NY, USA), 10 µg/mL of EGF, 5 µg/mL of insulin, 4 pg/mL Triiodothyronine (T3), 36ng/mL of hydrocortisone. After 24 hours of FCS starvation, HK-2 cells were transfected with 5 nmol IDT® miRNA inhibitor targeting hsa-miR-145-5p (Exiqon miRCURY LNA Inhibitor, sequence: 5'-CGATTCCTGGGAAAACTGGA-3') or with scrambled siRNA (Exiqon miRCURY LNA Inhibitor Control, sequence: 5'-TAACACGTCTATACGCCCA-3'), using the DharmaFECT Duo transfection reagent (Dharmacon, Lafayette, CO, USA).

Protein extraction and GeLC-MS sample preparation protocol

For protein extraction, cell pellets were re-suspended in isoelectric focusing sample buffer containing 7M Urea (ApliChem Inc., Missouri, USA), 2M Thiourea (Fluka, Sigma Aldrich Co., St. Louis USA), 4% w/v CHAPS (ApliChem Inc., Missouri, USA), 1% w/v DTE, (Sigma Aldrich Co., St. Louis USA) and 2% v/v IPG ampholytes (BioRad Laboratories Inc., CA, USA). The cells were sonicated for 15 minutes in a bath sonicator followed by centrifugation at 16.000 rcf for 20 min at RT to collect the supernatants. Protease inhibitors 3.6% v/v (Roche) were added to the extracts and the protein concentration was determined by Bradford assay (BioRad Laboratories Inc., CA, USA).

Next, 10 µg of the sample were loaded on SDS PAGE (5% stacking, 12% separating) and the electrophoresis was stopped when the samples just entered the separating gel. The gels were fixed with 30% v/v Methanol (Fisher) 10% v/v Acetic Acid for 30 min at room temperature followed by wash with water (2x10 min washes). Gels were stained with coomassie colloidal blue stain o/n at RT. After

washing the gels with water, the protein bands were excised from the gels and were sliced in small pieces (1-2mm). Gel pieces were destained with 40% Acetonitrile, 50mM NH_4HCO_3, reduced with 10mM DTE in 100mM NH_4HCO_3 for 20 min at room temperature,and alkylated with 10mg/mL Iodoacetamide in 100mM NH_4HCO_3 for 20 min at room temperature, in the dark. After alkylation the samples were washed with 100mM NH_4HCO_3 followed by another wash with 40% Acetonitrile, 50mM $NH_4HCO_3$and a final wash with ultra pure water was performed(20 min, at RT for each wash). Gel pieces were dried in a centrifugal vacuum concentrator (speed vac). Trypsinization was performed o/n at RT in the dark. 600ng of trypsin was added per sample (10ng/μL tryspin stock solution in 10mM NH_4HCO_3, pH 8.5). Finally, the peptides were extracted after incubation with 50mM NH_4HCO_3 for 15min, RT, followed by two incubations with 10% Formic Acid, Acetonitrile (1:1) for 15 min, RT. The peptide solution was filtered with PVDF filters (Merck Millipore) and was dried in a centrifugal vacuum concentrator. Samples were stored at -20^o C until further use.

LC–MS/MS analysis

2.5 μg of protein digest were loaded onto a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK). After loading onto a Dionex 0.1×20 mm 5 μm C18 nano trap column at a flow rate of 5 μl/min in 0.1% formic acid and 2% acetonitrile, samples were applied onto an Acclaim PepMap C18 nano column 75 μm×50 cm (Dionex, Sunnyvale, CA, USA), 2 μm 100 Å at a flow rate of 0.3 μl/min. The trap and nano flow column were maintained at 35°C. The samples were eluted with a gradient of solvent A: 0.1% formic acid versus solvent B: 80% acetonitrile starting at 1% B for 5 min rising to 5% B at 10 min then to 25% B at 180 min and 45% B at 240 min. The column was then washed and re-equilibrated prior to injection of the next sample. The eluent was ionized using a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). Ionization voltage was 2.8 kV and the capillary temperature was 250°C. The mass-spectrometer was operated in MS/MS mode scanning from 380 to 1,600 amu. The resolution of ions in MS1 was 60,000 and 15,000 for HCD MS2. The top 20 multiply charged ions were selected from each scan for MS/ MS analysis using HCD at 40% collision energy. AGC settings were 1,000,000 for full scan in the FTMS and 50,000 for MSn.

Data processing

Protein identification was performed using the SEQUEST search engine **(Eng et al., 1994)** (Proteome Discoverer 1.4, Thermo Scientific). Protein search was performed against the SwissProt human protein database **(Bairoch and Apweiler, 2000)** (30.05.2016) containing 20197 entries without protein isoforms. Trypsin was set as protease and two missed cleavage sites were allowed. Precursor mass tolerance was set to 10ppm and fragment mass tolerance to 0.05Da. Oxidation of methionine and carbamidomethylation of cysteine were set as variable and fixed modifications respectively. False discovery rate (FDR) was estimated with the percolator validation node included in the Proteome

Discoverer software package. FDR validation was based on q-value. Peptide identifications with false discovery rate >1% were discarded. Peptide rank was set to 1.

Bioinformatics analysis

Pathway analysis of the NGS data were performed using Ingenuity Pathway Analysis (IPA) software version 24390178 (IPA®, QIAGEN Redwood City, see http://www.Ingenuity.com). Statistical analysis was performed using GraphPad Prism v.5.01.

5.5 RESULTS

Next Generation Sequencing (NGS) analysis identifies multiple urinary miRNAs associated to CKD progression

NGS analysis of the urinary miRNA content was obtained from 35 matched patients with progressive CKD (≥3.3% reduction in eGFR slope/year) and 35 patients with non-progressive CKD (≤2% reduction and ≤4.5% increase in eGFR slope/year, **Table 1, Supplementary Table 1A**). When comparing the two groups, we identified 91 miRNAs with significantly ($p<0.05$) different urinary levels. Upon correction for multiple testing, 24 of the 91 remained significant (adjusted p-value of $p<0.05$) **(Table 3, Supplementary Table 2)**. Eighteen miRNAs displayed increased and 6 decreased urinary abundance in progressors compared to non-progressors **(Table 3)**.

Table 3: List of the 24 miRNAs detected with NGS with significant difference (adj. Pvalue < 0.05) between progressors and non-progressors.

miRBase	Fold change (progressors/ non-progressors)	Unadjusted p-value	Adjusted p-value
hsa-miR-34c-5p	8,743	4,56E-21	3,16E-18
hsa-miR-3200-5p	35,233	2,87E-09	0,000001
hsa-miR-944	9,068	2,26E-08	0,000005
hsa-miR-34b-3p	39,715	0,000001	0,000203
hsa-miR-147b	0,727	0,000005	0,000586
hsa-miR-410-3p	1,734	0,000005	0,000586
hsa-miR-513c-5p	Only in progressors	0,000007	0,000662
hsa-miR-3928-3p	15,575	0,000017	0,001463
hsa-miR-1289	Only in non-progressors	0,000027	0,001866
hsa-miR-1323	Only in non-progressors	0,000027	0,001866
hsa-miR-324-5p	0,301	0,000039	0,002478
hsa-miR-3150a-3p	38,655	0,000072	0,004136
hsa-miR-892c-3p	Only in progressors	0,000104	0,005527
hsa-miR-369-3p	4,228	0,000119	0,005891
hsa-miR-301b-3p	3,377	0,000381	0,017592
hsa-miR-449b-5p	3,815	0,000406	0,017592
hsa-miR-651-5p	6,723	0,000548	0,022325
hsa-miR-561-5p	1,198	0,000727	0,026699
hsa-miR-708-5p	0,809	0,000732	0,026699
hsa-miR-323b-3p	0,717	0,000896	0,029581
hsa-miR-493-5p	15,238	0,000891	0,029581
hsa-miR-487b-3p	4,172	0,001150	0,036223
hsa-miR-145-5p	4,850	0,001387	0,041795
hsa-miR-508-3p	3,241	0,001559	0,045015

Identified proteins and differential expression analysis

The 70 urine samples (35 progressors and 35 non progressors) were analyzed by high-resolution mass spectrometry and the resulting spectra were processed by Proteome Discoverer. After processing of the data by in-house software and ensuring of peptide consistency (as described in the Materials and Methods section), 344 proteins were found reproducibly identified and considered for differential expression analysis (**Supplementary Table 3**).

A protein was considered as differentially expressed if its corrected p-value was ≤ 0.05 and its progressors/non-progressors ratio was ≤ 0.66 or ≥ 1.5. This resulted in identification of 142 differentially secreted proteins, from which 34 were up-regulated and 108 down-regulated in CKD progressors compared to non-progressors (**Supplementary table 3**). Up regulated proteins included mainly highly-abundant plasma proteins (e.g. albumin, serotransferrin, vitamin D-binding protein), while down regulated proteins were more often related to biologically relevant functions in the context of CKD including inhibitors, receptors, peptidases and proteases (e.g. tumor necrosis factor receptor, plasma serine protease inhibitor, carboxypeptidase).

Pathway analysis enrichment revealed a relation with kidney pathology

Pathway enrichment analysis of the 24 miRNAs and 142 proteins revealed that 7 miRNAs are predicted to regulate 15 proteins in molecular pathways including Acute Phase Response Signaling, Epithelial Adherens Junction Signaling, Regulation of the Epithelial-Mesenchymal Transition Pathway, TGF-β Signaling, Wnt/β-catenin Signaling, NF-κB Signaling and ILK Signaling and others that are linked with kidney pathology (**Supplemmentary Table 4**).

When focusing on involvement in pathological mechanisms, miR-34c-5p, miR-145-5p and miR-301b-3p together with some of their targets showed to be potentially involved in kidney damage, including growth of epithelial tissue, differentiation and proliferation of the cells, apoptosis, necrosis and cell death **(Supplementary Table 5)**.

TaqMan low-density array analysis (TLDA) validates urinary miR-145-5p as associated to progression of CKD

For the validation phase, we selected 4 from the 7 miRNAs that were predicted to target at least 2 proteins to increase the stringency of the selection. These miRNAs were miR-34c-5p, miR-145-5p, miR-301b-3p and miR-401-5p (**Table 4).** The urinary abundance of the 4 selected miRNAs was evaluated in an independent cohort of 52 patients with CKD using TLDA (**Table 2, Supplementary Table 1B)**. We also included in the analysis 1) two frequently used miRNAs for normalization in the literature, U6 snRNA and RNU48 and 2) the urinary miRNA showing the best normalization potential in CKD patients, hsa-miR-30a-5p, as it was detected with highest frequency (in all 70 samples) and displayed highest stability (smallest fold change between the groups) (**Supplementary Table 2)**.

Table 4: The 4 miRNAs targeting at least 2 proteins that were selected for validation.

Symbol	Exp Fold Change	Symbol	Exp Fold Change
miR-301b-3p	3.377	EPHB4	-1.586
		IL6ST	-3.173
		LDLR	-1.671
		PTPRG	-2.078
		TNFRSF1B	-1.587
miR-145-5p	4.850	ACVR1B	-2.278
		CDH2	-2.738
		EGF	-2.788
		MUC1	-3.156
		NECTIN1	-5.588
miR-410-3p	1.734	FZD2	-1.584
		LDLR	-1.671
miR-34c-5p	8.743	NECTIN1	-5.588
		PDGFRB	-1.921
		SERPINF2	-1.640

Analysis of the 3 miRNAs selected for normalization showed that RNU48 could not serve as normalizing miRNA since it was absent in 15 out of 52 samples (**Figure 1B**). In contrast, U6 and hsa-miR-30a-5p were detected in all samples (**Figure 1A and 1C**) with miR-30a-5p displaying the most stable expression pattern and hence was chosen for the normalization of the data.

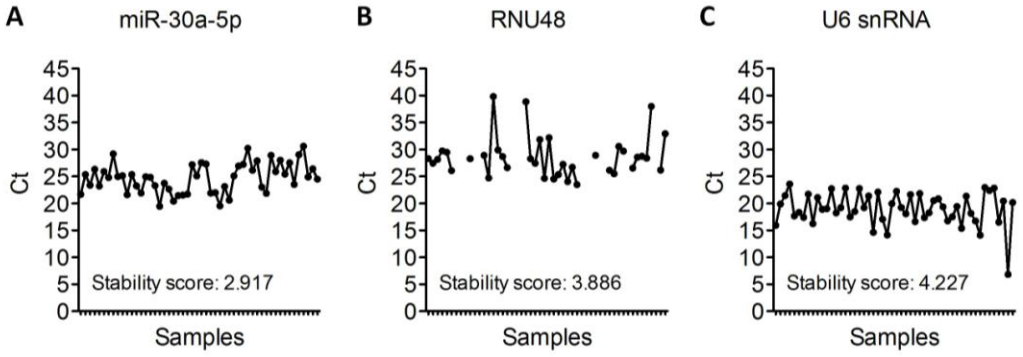


Figure 1: Assessment of miRNAs stability for normalization. Normalization plots were generated for A) hsa-miR-30a-5p, B) U6 snRNA and C) RNU48. Stability of miRNAs was assessed by calculation of a stability score using ExpressionSuite software (Applied Biosystems), the lower the score being associated with the more stable expression of the given miRNA relative to all other miRNAs in the comparison.

From the 4 miRNAs selected for validation, miR-410-3p and miR-301b-3p were detected with very low frequency in the 52 samples (**Figure 2**). MiR-34c-5p was detected in nearly 20% of the samples but did not display a statistically significant difference between progressors and non-progressors (**Figure 2**). However, miR-145-5p was detected in 44% of the samples in the validation set and could be validated as significantly associated to CKD progression with a fold change close to the one observed in the discovery cohort (3.151 versus 4.850) (**Figure 2**).

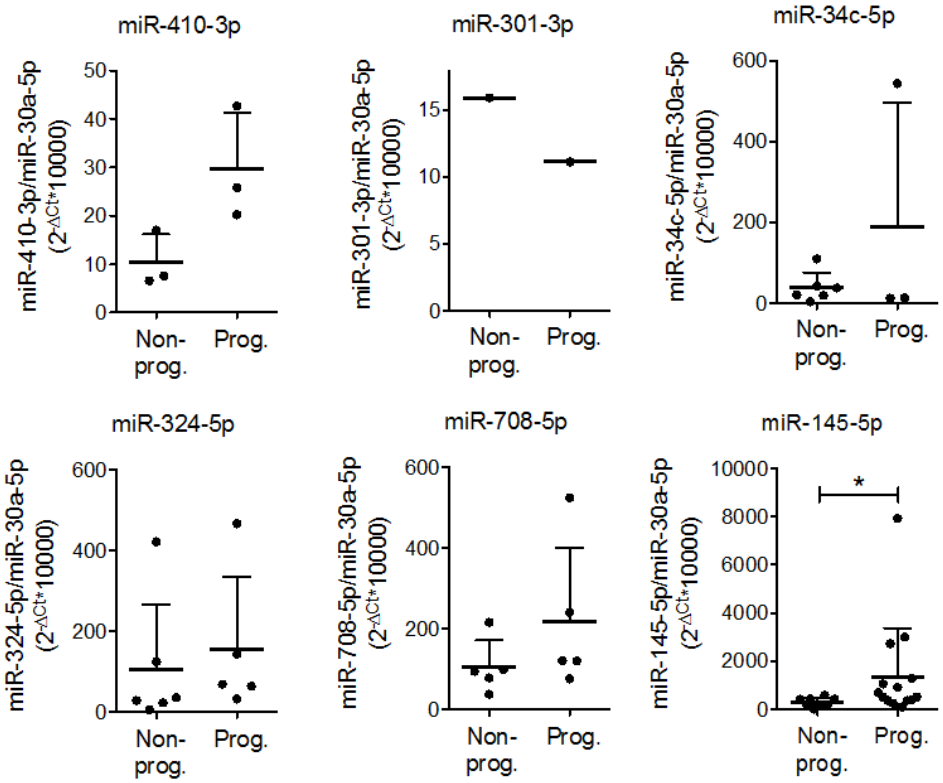


Figure 2: Boxplots of the delta Cts (mean + SD) of the six miRNAs studied with TLDA analysis in the validation cohort. In dots the values of the individual samples. Only miR-145-5p scored significantly different between the two groups under study. Normalization with miR-30a-5p. * pvalue < 0.05.

Interestingly, urinary miR-145-5p abundance was not only associated with CKD progression, it was also slightly but significantly inversely correlated to eGFR decline in CKD patients from the validation cohort (Spearman r=-0.5, p>0.05, **Figure 3A**), and showed a moderate predictive value with an AUC of 0.733 **(Figure 3B)**.

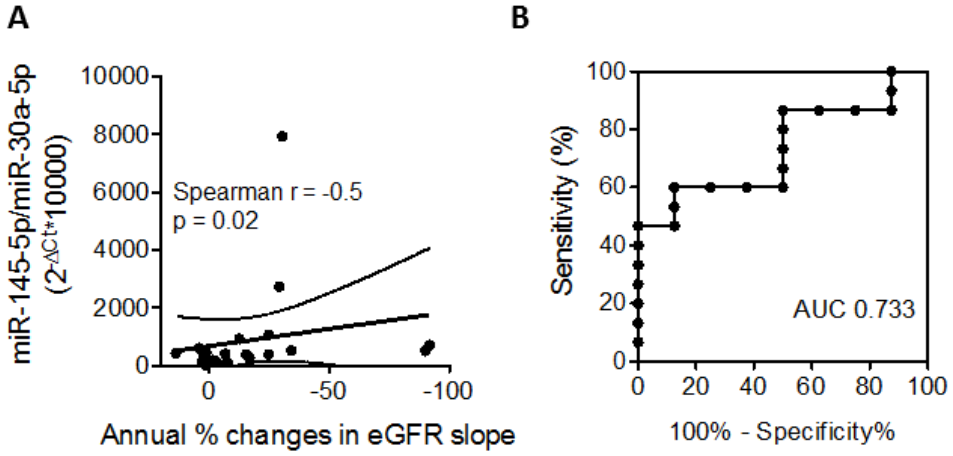


Figure 3: A) Spearman correlation showing the correlation between the increase of miR-145-5p and the annual % change in eGFR slope. B) ROC curve showing a possible predictive value of miR-145-5p.

Proteome analysis of human kidney cells treated with a hsa-miR-145-5p antagomir reveals a possible role in necrosis

In order to determine the effect of the alteration of miR-145-5p expression, we performed an independent study using human kidney cells. Human kidney cells (HK2) were treated with an antagomir of hsa-miR-145-5p in order to inactivate the function of miR-145-5p and study the effect of this inactivation on the proteome level. HK2 cells treated with a scrambled antagomir were used as a control. LC-MS/MS analysis of the two conditions (5 samples treated with antagomir for miR-145 and 5 treated with scrambled antagomir) allowed the identification of 26 high confidence proteins with significantly different levels (non-adjusted pvalues) between the two conditions. Twelve proteins were up- and 14 proteins were down-regulated in the cells treated with the antagomir compared to control (**Table 5).** Pathway analysis of the 26 proteins with revealed cell death and necrosis as the most significantly activated mechanism (p-value 1.28E-07 and activation z-score 0.203) in response to inactivation of miR-145-5p, in particular through modulation of the expression of splicing factor U2AF 65 kDa subunit (U2AF2), probable ATP-dependent RNA helicase DDX17 (DDX17), nucleolin (NCL), DNA replication licensing factor MCM7 (MCM7) and transcription intermediary factor 1-beta (TRIM28).

Table 5: Twenty-six significantly different proteins in HK-2 cell line treated with antagomir against has-miR-145-5p versus cells treated with the scrambled antagomir. In bold the proteins both related to necrosis and that are predicted to be miR-145-5p targets.

Symbol	Name	Fold change (ant-miR-145/control)	p-value Mann-Whitney
SNX3	Sorting nexin-3	Only in ant-miR-145	0,0254
PAICS	Multifunctional protein ADE2	Only in ant-miR-145	0,0254
NAMPT	Nicotinamide phosphoribosyltransferase	Only in ant-miR-145	0,0254
GDI2	Rab GDP dissociation inhibitor beta	8,13	0,0449
PNP	Purine nucleoside phosphorylase	6,85	0,0449
U2AF2	**Splicing factor U2AF 65 kDa subunit**	**5,96**	0,0449
PRPS1	Ribose-phosphate pyrophosphokinase 1	2,87	0,0367
DDX17	**Probable ATP-dependent RNA helicase DDX17**	**2,86**	0,0212
IMPDH2	Inosine-5'-monophosphate dehydrogenase 2	1,91	0,0367
VDAC2	Voltage-dependent anion-selective channel protein 2	1,76	0,0122
CAPG	Macrophage-capping protein	1,51	0,0367
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	1,41	0,0122
LGALS1	Galectin-1	0,70	0,0367
LMNA	Prelamin-A/C	0,69	0,0122
NCL	**Nucleolin**	**0,68**	0,0216
RPS19	40S ribosomal protein S19	0,44	0,0361
UBA52	Ubiquitin-60S ribosomal protein L40	0,43	0,0367
MCM7	**DNA replication licensing factor MCM7**	**0,35**	0,0122
MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	0,35	0,0212
YWHAG	14-3-3 protein gamma	0,32	0,0112
TRIM28	**Transcription intermediary factor 1-beta**	**0,32**	0,0212
QPRT	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	0,13	0,0449
YWHAH	14-3-3 protein eta	0,11	0,0449
MCM4	DNA replication licensing factor MCM4	Only in control	0,0254
EPCAM	Epithelial cell adhesion molecule	Only in control	0,0254
DSTN	Destrin	Only in control	0,0254

5.6 DISCUSSION

CKD is now recognized as a global health and economical burden affecting millions of people worldwide **(Ayodele and Alebiosu, 2010, De Nicola and Minutolo, 2016)**. The limited understanding of the molecular mechanisms causing the disease and the lack of reliable noninvasive diagnostic and prognostic biomarkers complicates its management **(Ortiz, 2015)**. Earlier studies have shown that miRNAs can potentially contribute to the development of CKD or its progression **(Trionfini et al., 2015, Zhong et al., 2011, Qin et al., 2011)**. In addition, many studies have related urinary miRNAs with different kidney diseases, showing their potential to serve as non-invasive biomarkers **(Papadopoulos et al., 2015b)**.

In our retrospective study we used urinary samples from established CKD patients to investigate the association of urinary miRNAs to progressive CKD or non-progressive CKD. We were able to identify 24 miRNAs using Next Generation Sequencing that were observed to be significantly different between our two groups (adjusted pvalue<0.05) and associated to CKD progression. Combination of these 24 miRNAs with a dataset of 142 proteins found in significant different levels in the same urine samples, followed by miRNA target prediction showed that 7 miRNAs and 15 proteins may be connected and involved into pathways related to kidney damage in CKD and may associate to progression of CKD in the discovery population

Four miRNAs out of the 7 were further validated in an independent CKD patient population. From these 4 miRNAs, increased urinary abundance of miR-145-5p was confirmed to be associated to CKD progression. Previously, MiR-145 was mostly studied in tumors and is characterized as tumor suppressor miRNA **(Elmen et al., 2008a)**. MiR-145 has also been identified as a regulator of controlling smooth muscle cell phenotype and vascular formation **(Wiggins et al., 2010, Trang et al., 2011)**. Recent studies showed decrease of serum miR-145 in very early-onset coronary artery disease (CAD) patients compared to healthy controls **(Ebert et al., 2007, Horie et al., 2009)**. Furthermore, miR-145 was found to be increased in smooth muscle cells of patients with insulin resistance that can lead to type 2 diabetes mellitus (T2DM) **(Chistiakov et al., 2012).**

MiR-145 has also been previously found to be associated to (chronic) kidney disease. In a biopsy study of lupus nephritis patients, miR-145 expression in vascular smooth muscle cells (VSMC) was associated to different VSMC phenotypes. High expression level of miR-145 in the renal vasculature was associated with VSMCs of the contractile phenotype while a medium expression level was associated with VSMCs of the proliferative phenotype **(Chen et al., 2010)**. Moreover, miR-145 was found decreased in plasma of CKD patients with correlation to the progressive loss of eGFR **(Chen et al., 2015b)** and elevated in urinary exosomes of type 1

diabetic patients with incipient diabetic nephropathy **(Barutta et al., 2013, Argyropoulos et al., 2015)**. Finally, increased levels of serum miR-145 was found to correlate with CKD severity **(Brigant et al., 2016)**. These observations confirm a tight connection of miR-145 with CKD.

Pathway analysis revealed the possible involvement of miR-145-5p via its targets in molecular pathways and mechanisms present in kidney disease (**Supplementary table 4 and 5)**. In particular, cadherin-2 (CDH2) and epithelial growth factor (EGF) are proteins involved in epithelial adherens junction signaling, in regulation of the epithelial-mesenchymal transition (EMT) Pathway, Wnt/β-catenin signaling and NF-κB signaling, pathways that are reported to be activated in kidney pathologies that can lead to renal fibrosis, apoptosis and necrosis and may lead to CKD **(Boussif et al., 1995, Chen et al., 2015b, Zhang et al., 2013, Ghosh et al., 2013, Lu et al., 2005, Kumar et al., 2009, Blenkiron et al., 2007, Faruq and Vecchione, 2015, Takamizawa et al., 2004, Calin et al., 2005, Hayes et al., 2014)**. Finally, mucin-1 (MUC-1) a protein with role in EMT and in autosomal dominant tubulointerstitial kidney disease **(Pan et al., 2010)**, may be part of ILK signaling pathway, a pathway with anti-inflammatory properties in renal inflammation **(Faruq and Vecchione, 2015, Lawrie et al., 2008)**.

An independent proteomics analysis was also performed in human kidney (HK2) cells treated with an antagomir against miR-145-5p followed by a bioinformatics analysis predicting the possible deregulation of 14 proteins associated with inhibition of necrosis and cell death, a feature seen in CKD progression in the form of acute tubular necrosis (ATN). ATN follows acute kidney injury and it is very common in patients with progressive CKD and to those who reached end-stage renal disease **(Zhang et al., 2010, Wu et al., 2011, Hsu et al., 2013)**. It must be noted, however, that none of the 14 proteins modified by the antagomer treatment in cells was detected among the 142 proteins associated to CKD progression. This is most likely explained by the fact that in patients the changes in protein abundance in urine are multifactorial (eg the CKD condition) and that in cells the only perturbant was the miR-145-5p antagomer.

From the 14 proteins related to necrosis inhibition mechanism, 5 were predicted to be targets of miR-145-5p with DIANA-microT web server (version 5.0) **(Paraskevopoulou et al., 2013)**. These proteins were Splicing factor U2AF (U2AF2), DEAD-Box Helicase 17 (DDX17), Nucleolin (NCL), DNA replication licensing factor MCM7 and Transcription intermediary factor 1-beta (TRIM28). DDX17 is a member of the DEAD-box protein family, which are putative RNA helicases involved in alteration of RNA secondary structure, leading to cellular growth and division **(Kulkarni et al., 1971)**. DDX17 has been found with increased abundance in the serum of patients suffering from IgA nephropathy and correlated to the progression of the disease **(Su et al., 2011).** MiR-145 has been reported to down regulate DDX17 in gut tumors in a mouse model

(Chen et al., 2015b). Nucleolin is proposed to be involved in renal tubular crystal retention via the form of an acidic fragment of nucleolin-related protein **(Panyam and Labhasetwar, 2003)** and, also, as an antibody target to reduce apoptosis and endothelial cell proliferation in renal allograft rejection **(Blum and Saltzman, 2008)**. Finally, in an early stage of kidney embryonic development Trim28 is highly expressed in the cap mesenchyme and at the tips of the ureteric bud enforcing the development of branches, while in developed kidney it is of minor abundance. Experiments of knocking down Trim28 resulted in branching arrest suggest that Trim28 plays an important role in kidney branching and morphogenesis **(Zhang et al., 2013)**. For MCM7 and U2AF2 proteins no reports in association to kidney physiology or pathology exists.

This study also represented a number of technical challenges. To our knowledge, this is the first report to use Next Generation Sequencing (NGS) to analyze urinary miRNAs from a little as 500 µl of urine of archived urine samples and opens up the possibility to perform NGS in other (renal) diseases were such small amounts of urine is available. Another challenge is represented by the shift in technology between the discovery (NGS) and the validation (Taqman technology, a real-time qPCR based method) step which might have a negative effect on the number of miRNAs validated. In a recent study, Git *et al.* compared NGS and PCR and showed that the different preparation of the samples before applying the method, as well as the higher sensitivity of NGS and the variability of the samples themselves is responsible for producing different results between the two methods **(Hafner et al., 2010)**. The change in technology potentially only allowed to validate the miRNAs that were the most strongly associated to progression of CKD while the more weakly associated most likely need a larger number of samples in the validation set to become significant.

Overall, we identified urinary hsa-miR-145-5p to be associated with progressive CKD in patients from both the discovery and validation phase using two different technologies. Using *in vitro* knockdown studies the most likely mechanism linking mir-145-5p to CKD progression is necrosis. Further studies on larger sets of patients are warranted to confirm these associations.

5.7 Supplementary Data

Supplementary Table 1A: The clinical parameters of the discovery cohort (N=70)

Rflab code	Sample ID	Sex	Sex	Age	Diabetes	Diabetes	Follow-up duration [years]	timepoints	Disease Etiology	Outcome	First sampling date	Baseline Serum Creatinine (mg/dl)	Baseline eGFR [CKD-EPI]	Baseline CKD stage [CKD-EPI]	Linear coefficient	Linear interecpt	annual % slope changes in eGFR	State	Last follow-up date	Last follow-up serum creatinine [mg/dL]	Last_follow_up CKD-EPI	Last CKD stage	ACEi...ARB	Statins	Anticoagulation	Vitamin.D
iMODE 67	CKD_BIO_0102	M	2	45	No	1	2,3	2	Membranous n		14/02/2013	1,47	56,62	3	- 12,44	56,62	-22,0	Progressor	16/06/2015	2,58	28,00	4	1	0	0	0
iMODE 58	CKD_BIO_0108	F	1	51	No	1	1,8	2	Minimal change		19/03/2013	0,88	75,93	2	- 1,09	75,93	1,4	Non-progressor	24/12/2014	0,85	77,90	2	0	0	0	0
iMODE 59	CKD_BIO_0112	M	2	64	No	1	1,8	2	Membranous n		18/04/2013	0,90	89,63	2	- 15,21	89,63	-17,0	Progressor	03/02/2015	1,20	62,26	2	1	0	0	0
iMODE 61	CKD_BIO_0114	M	2	44	No	1	2	9	Focal segmenta		24/05/2013	1,18	74,93	2	- 37,08	78,73	-49,5	Progressor	10/06/2015	7,13	8,37	5	1	0	0	0
iMODE 55	CKD_BIO_0209	F	1	40	No	1	1,5	4	Glomerulonepl		18/04/2013	1,00	70,78	2	- 4,81	72,19	-6,8	Progressor	13/10/2014	1,10	62,27	2	1	1	0	1
iMODE 53	CKD_BIO_0212	F	1	52	No	1	1,3	4	Transplant glon		26/02/2013	1,00	65,19	2	- 11,05	64,02	-16,9	Progressor	02/07/2014	1,20	51,57	3	1	1	0	0
iMODE 57	CKD_BIO_0305	F	1	41	No	1	1,6	4	IgA nephropath		17/07/2013	1,43	45,71	3	0,45	46,60	1,0	Non-progressor	11/03/2015	1,37	47,45	3	1	0	0	0
iMODE 51	CKD_BIO_0307	M	2	53	No	1	1,3	3	IgA nephropath		05/04/2013	1,12	74,53	2	1,92	70,48	2,6	Non-progressor	15/07/2014	1,11	74,77	2	0	0	0	0
iMODE 41	CKD_BIO_0309	F	1	61	No	1	0,3	2	Focal segmenta		04/06/2014	0,74	88,18	2	- 25,81	88,18	-29,3	Progressor	03/10/2014	0,79	80,43	2	1	0	0	0
iMODE 65	CKD_BIO_0310	M	2	27	No	1	2,3	5	IgA nephropath		18/06/2013	1,22	80,72	2	1,54	79,39	1,9	Non-progressor	14/10/2015	1,15	85,09	2	1	0	0	0
iMODE 64	CKD_BIO_0315	M	2	45	No	1	2,2	5	IgA nephropath		10/05/2013	1,81	44,14	3	0,80	49,03	1,8	Non-progressor	07/07/2015	1,63	49,38	3	0	0	0	0
iMODE 46	CKD_BIO_0503	M	2	55	No	1	1	2	Membranous N		10/04/2014	1,39	56,49	3	7,25	56,49	-12,8	Progressor	15/04/2015	1,55	49,24	3	1	0	0	1
iMODE 69	CKD_BIO_1102	F	1	63	Yes	2			Diabetic nephro	dialysis	01/07/2013	2,14	23,91	4				Progressor					0	0	0	0
iMODE 62	CKD_BIO_1108	F	1	84	Yes	2	2,1	3	Diabetic nephro		15/08/2013	2,81	14,91	5	- 1,16	14,96	-7,8	Progressor	08/09/2015	3,27	12,22	5	1	0	0	0
iMODE 54	CKD_BIO_1109	F	1	20	No	1	1,5	2	Focal segmenta	ESRD	26/08/2013	3,25	19,58	4	- 7,72	19,58	-39,4	Progressor	09/02/2015	6,75	7,99	5	1	0	0	0
iMODE 56	CKD_BIO_1122	M	2	77	No	1	1,6	4	Membranous n		08/01/2014	1,58	41,46	3	- 8,69	41,06	-21,0	Progressor	17/08/2015	2,09	29,26	4	1	0	0	0
iMODE 66	CKD_BIO_1501	M	2	44	No	1	2,3	5	Focal segmenta		28/03/2012	1,63	50,52	3	- 7,91	56,41	-15,6	Progressor	24/07/2014	2,10	36,47	3	0	1	0	0
iMODE 68	CKD_BIO_1502	M	2	65	No	1	2,4	6	IgA Nephropath		22/06/2012	2,41	27,21	4	- 0,16	22,85	-0,6	Non-progressor	19/11/2014	2,58	24,64	4	0	1	0	0
iMODE 60	CKD_BIO_1503	M	2	69	No	1	2	5	Membranous N		26/09/2012	1,21	60,55	2	- 18,69	62,55	-30,9	Progressor	10/09/2014	2,36	26,58	4	1	0	0	0
iMODE 50	CKD_BIO_2002	M	2	49	No	1	1,3	3	Membranous n		07/07/2014	1,23	68,33	2	- 17,08	69,72	-25,0	Progressor	09/10/2015	1,80	42,91	3	1	1	1	0
iMODE 49	CKD_BIO_2003	M	2	45	No	1	1,2	3	Focal segmenta		15/07/2014	1,99	39,35	3	- 6,19	39,07	-15,7	Progressor	25/09/2015	2,29	33,04	3	0	1	0	0
iMODE 45	CKD_BIO_2004	M	2	71	No	1	0,6	2	Acute kidney in		10/09/2014	2,90	20,80	4	- 7,16	20,80	-34,4	Progressor	06/04/2015	3,50	16,50	4	0	1	0	0
iMODE 52	CKD_BIO_2103	M	2	72	No	1	1,3	10	IgA nephropath		27/06/2014	3,73	15,20	4	- 6,47	17,46	-42,6	Progressor	23/10/2015	5,11	10,30	5	1	1	1	1
iMODE 47	CKD_BIO_2105	M	2	19	No	1	1	3	Mesangiocapill		14/08/2014	1,20	87,12	2	- 26,77	92,41	-30,7	Progressor	21/10/2015	1,73	55,44	3	1	0	0	1
iMODE 42	CKD_BIO_2111	F	1	69	No	1	0,5	2	Mesangial proli		11/03/2015	1,06	53,50	3	- 48,28	53,50	-90,2	Progressor	24/08/2015	1,74	29,36	4	1	0	0	0
iMODE 44	CKD_BIO_2305	M	2	28	No	1	0,5	2	Focal segmenta		27/04/2015	2,19	39,40	3	- 17,77	39,40	-45,1	Progressor	13/10/2015	2,70	30,52	3	1	0	0	0
iMODE 70	CKD_BIO_2309	M	2	75	No	1			Focal segmenta	dialysis	27/04/2015	3,57	15,70	4				Progressor					0	0	0	0
iMODE 43	CKD_BIO_2310	M	2	54	No	1	0,5	2	Mesangial proli		27/04/2015	1,82	41,02	3	- 37,68	41,02	-91,8	Progressor	10/10/2015	3,02	22,18	4	1	0	0	0
iMODE 11	UZ_GENT_0022	M	2	79	Yes	2	3,1	16	Diabetic nephro		27/01/2011	2,78	20,56	4	- 0,93	22,40	-4,5	Progressor	07/03/2014	3,09	17,71	4	1	1	0	1
iMODE 34	UZ_GENT_0062	F	1	53	No	1	2,5	4	IgA nephropath		28/02/2011	0,84	79,61	2	1,84	76,03	2,3	Non-progressor	22/08/2013	0,80	82,68	2	1	0	0	0
iMODE 13	UZ_GENT_0120	M	2	82	No	1	2,9	9	Chronic hyperte		31/03/2011	1,42	45,67	3	- 2,16	47,61	-4,7	Progressor	25/02/2014	1,44	43,97	3	0	1	0	0
iMODE 31	UZ_GENT_0122	M	2	79	Yes	2	2,8	17	Granulomatosis		23/03/2011	1,45	45,48	3	0,62	44,37	1,4	Non-progressor	07/01/2014	1,53	41,73	3	1	0	0	0
iMODE 36	UZ_GENT_0149	F	1	67	No	1	3,1	11	Primary focal se		19/04/2011	1,06	54,46	3	1,43	51,86	2,6	Non-progressor	22/05/2014	1,03	55,21	3	1	1	0	1
iMODE 12	UZ_GENT_0173	M	2	68	No	1	2,9	19	Chronic hyperte		29/04/2011	1,64	42,34	3	- 1,64	40,57	-3,9	Progressor	03/04/2014	1,82	36,55	3	1	1	0	0
iMODE 15	UZ_GENT_0177	M	2	35	Yes	2	3	10	Adult nephrotic		03/05/2011	1,13	83,74	2	- 2,94	78,70	-3,5	Progressor	08/05/2014	1,27	71,20	2	1	0	0	0
iMODE 21	UZ_GENT_0218	F	1	56	No	1	3	7	Systemic lupus		26/05/2011	0,71	95,52	1	- 1,74	85,80	-1,8	Non-progressor	05/06/2014	0,75	87,53	2	0	1	0	1
iMODE 7	UZ_GENT_0226	F	1	70	No	1	3	27	Systemic vascul		06/06/2011	3,13	14,40	5	- 1,14	14,47	-7,9	Progressor	02/06/2014	4,15	10,03	5	0	1	0	1
iMODE 39	UZ_GENT_0321	M	2	72	No	1	2,7	12	Chronic hyperte		30/08/2011	1,90	34,45	3	1,32	35,96	3,8	Non-progressor	21/05/2014	1,95	32,69	3	0	0	0	0
iMODE 30	UZ_GENT_0329	M	2	50	Yes	2	2,5	5	Acute kidney in		07/09/2011	1,23	68,02	2	0,62	74,08	0,9	Non-progressor	20/02/2014	1,14	73,01	2	1	1	0	0
iMODE 28	UZ_GENT_0333	M	2	54	No	1	2,8	11	IgA nephropath		08/09/2011	1,40	56,56	3	2,44	53,85	4,3	Non-progressor	12/06/2014	1,32	59,88	3	1	0	0	1
iMODE 27	UZ_GENT_0385	M	2	67	No	1	2,5	21	Chronic hyperte		14/10/2011	1,52	46,74	3	- 0,22	43,00	-0,5	Non-progressor	10/04/2014	1,86	35,85	3	1	1	1	0
iMODE 4	UZ_GENT_0386	M	2	76	Yes	2	2,1	14	Diabetic nephro		14/10/2011	1,03	70,23	2	- 2,97	58,68	-4,2	Progressor	03/12/2013	1,13	61,91	2	0	1	1	1
iMODE 3	UZ_GENT_0415	F	1	42	No	1	2,3	16	Systemic lupus		01/12/2011	1,66	37,74	3	- 4,21	41,00	-11,2	Progressor	18/03/2014	1,65	37,23	3	1	0	0	1
iMODE 33	UZ_GENT_0427	M	2	25	No	1	2,5	16	Haemolytic ura		14/12/2011	1,26	78,76	2	2,18	81,88	2,8	Non-progressor	16/06/2014	1,22	80,75	2	1	0	0	0
iMODE 38	UZ_GENT_0437	M	2	72	Yes	2	2,4	9	Diabetic nephro		22/12/2011	1,84	35,82	3	0,42	34,47	1,2	Non-progressor	23/05/2014	1,90	33,73	3	1	0	0	0
iMODE 5	UZ_GENT_0448	M	2	68	Yes	2	2,1	18	Diabetic nephro		10/01/2012	1,07	70,95	2	- 3,36	64,25	-4,7	Progressor	04/02/2014	1,24	58,53	3	1	1	0	0
iMODE 1	UZ_GENT_0458	F	1	73	Yes	2	2,1	8	Diabetic nephro		26/01/2012	2,59	17,73	4	- 4,41	22,21	-24,9	Progressor	20/02/2014	3,30	13,04	5	1	1	0	1
iMODE 22	UZ_GENT_0463	M	2	53	No	1	2	3	Acute kidney in		03/02/2012	1,01	84,52	2	- 1,57	82,39	-1,9	Non-progressor	03/02/2014	1,03	81,39	2	1	1	0	0
iMODE 6	UZ_GENT_0478	M	2	67	No	1	2,1	13	Acute kidney in		24/02/2012	1,96	34,37	3	- 1,59	29,19	-4,6	Progressor	14/03/2014	2,74	22,44	4	0	1	1	0
iMODE 25	UZ_GENT_0506	F	1	80	Yes	2	2,2	27	Diabetic nephro		23/03/2012	1,79	26,38	4	3,59	18,04	13,6	Non-progressor	10/06/2014	1,82	25,50	4	0	0	0	1
iMODE 35	UZ_GENT_0533	M	2	79	No	1	2	5	Henoch-Schönl		14/05/2012	1,35	49,58	3	1,14	49,36	2,3	Non-progressor	19/05/2014	1,28	52,14	3	1	0	0	1
iMODE 2	UZ_GENT_0543	M	2	62	No	1	2	9	IgA nephropath		01/06/2012	3,91	15,45	4	- 1,97	15,22	-12,8	Progressor	23/05/2014	4,92	11,54	5	1	0	0	0

Supplementary Table 1B: The clinical parameters of the validation cohort (N=52)

Rflab code	Sample ID	Sex	Sex	Age	Diabetes	Diabetes	Follow-up duration [years]	timepoints	Disease Etiology	Outcome	First sampling date	Baseline Serum Creatinine (mg/dl)	Baseline eGFR [CKD-EPI]	Baseline CKD stage [CKD-EPI]	Linear coefficient	Linear interecpt	annual % slope changes in eGFR	State	Last follow-up date	Last follow-up serum creatinine [mg/dL]	Last_follow_up CKD-EPI	Last CKD stage	ACEi...ARB	Statins	Anticoagulation	Vitamin.D
iMODE 67	CKD_BIO_0102	M	2	45	No	1	2,3	2	Membranous n		14/02/2013	1,47	56,62	3	- 12,44	56,62	-22,0	Progressor	16/06/2015	2,58	28,00	4	1	0	0	0
iMODE 58	CKD_BIO_0108	F	1	51	No	1	1,8	2	Minimal change		19/03/2013	0,88	75,93	2	1,09	75,93	1,4	Non-progresso	24/12/2014	0,85	77,90	2	0	0	0	0
iMODE 59	CKD_BIO_0112	M	2	64	No	1	1,8	2	Membranous n		18/04/2013	0,90	89,63	2	- 15,21	89,63	-17,0	Progressor	03/02/2015	1,20	62,26	2	1	0	0	0
iMODE 61	CKD_BIO_0114	M	2	44	No	1	2	9	Focal segmenta		24/05/2013	1,18	74,93	2	- 37,08	78,73	-49,5	Progressor	10/06/2015	7,13	8,37	5	1	0	0	0
iMODE 55	CKD_BIO_0209	F	1	40	No	1	1,5	4	Glomerulonepl		18/04/2013	1,00	70,78	2	- 4,81	72,19	-6,8	Progressor	13/10/2014	1,10	62,27	2	1	1	0	1
iMODE 53	CKD_BIO_0212	F	1	52	No	1	1,3	4	Transplant glon		26/02/2013	1,00	65,19	2	- 11,05	64,02	-16,9	Progressor	02/07/2014	1,20	51,57	3	1	1	0	0
iMODE 57	CKD_BIO_0305	F	1	41	No	1	1,6	4	IgA nephropath		17/07/2013	1,43	45,71	3	0,45	46,60	1,0	Non-progresso	11/03/2015	1,37	47,45	3	1	0	0	0
iMODE 51	CKD_BIO_0307	M	2	53	No	1	1,3	3	IgA nephropath		05/04/2013	1,12	74,53	2	1,92	70,48	2,6	Non-progresso	15/07/2014	1,11	74,77	2	0	0	0	0
iMODE 41	CKD_BIO_0309	F	1	61	No	1	0,3	2	Focal segmenta		04/06/2014	0,74	88,18	2	- 25,81	88,18	-29,3	Progressor	03/10/2014	0,79	80,43	2	1	0	0	0
iMODE 65	CKD_BIO_0310	M	2	27	No	1	2,3	5	IgA nephropath		18/06/2013	1,22	80,72	2	1,54	79,39	1,9	Non-progresso	14/10/2015	1,15	85,09	2	1	0	0	0
iMODE 64	CKD_BIO_0315	M	2	45	No	1	2,2	5	IgA nephropath		10/05/2013	1,81	44,14	3	0,80	49,03	1,8	Non-progresso	07/07/2015	1,63	49,38	3	0	0	0	0
iMODE 46	CKD_BIO_0503	M	2	55	No	1	1	2	Membranous N		10/04/2014	1,39	56,49	3	- 7,25	56,49	-12,8	Progressor	15/04/2015	1,55	49,24	3	1	0	0	1
iMODE 69	CKD_BIO_1102	F	1	63	Yes	2			Diabetic nephr	dialysis	01/07/2013	2,14	23,91	4				Progressor					0	0	0	0
iMODE 62	CKD_BIO_1108	F	1	84	Yes	2	2,1	3	Diabetic nephr		15/08/2013	2,81	14,91	5	- 1,16	14,96	-7,8	Progressor	08/09/2015	3,27	12,22	5	1	0	0	0
iMODE 54	CKD_BIO_1109	F	1	20	No	1	1,5	2	Focal segmenta	ESRD	26/08/2013	3,25	19,58	4	- 7,72	19,58	-39,4	Progressor	09/02/2015	6,75	7,99	5	1	0	0	0
iMODE 56	CKD_BIO_1122	M	2	77	No	1	1,6	4	Membranous n		08/01/2014	1,58	41,46	3	- 8,69	41,06	-21,0	Progressor	17/08/2015	2,09	29,26	4	1	0	0	0
iMODE 66	CKD_BIO_1501	M	2	44	No	1	2,3	5	Focal segmenta		28/03/2012	1,63	50,52	3	- 7,91	56,41	-15,6	Progressor	24/07/2014	2,10	36,47	3	0	1	0	0
iMODE 68	CKD_BIO_1502	M	2	65	No	1	2,4	6	IgA Nephropath		22/06/2012	2,41	27,21	4	- 0,16	22,85	-0,6	Non-progresso	19/11/2014	2,58	24,64	4	0	1	0	0
iMODE 60	CKD_BIO_1503	M	2	69	No	1	2	5	Membranous N		26/09/2012	1,21	60,55	2	- 18,69	62,55	-30,9	Progressor	10/09/2014	2,36	26,58	4	1	0	0	0
iMODE 50	CKD_BIO_2002	M	2	49	No	1	1,3	3	Membranous n		07/07/2014	1,23	68,33	2	- 17,08	69,72	-25,0	Progressor	09/10/2015	1,80	42,91	3	1	1	1	0
iMODE 49	CKD_BIO_2003	M	2	45	No	1	1,2	3	Focal segmenta		15/07/2014	1,99	39,35	3	- 6,19	39,07	-15,7	Progressor	25/09/2015	2,29	33,04	3	0	1	0	0
iMODE 45	CKD_BIO_2004	M	2	71	No	1	0,6	2	Acute kidney in		10/09/2014	2,90	20,80	4	- 7,16	20,80	-34,4	Progressor	06/04/2015	3,50	16,50	4	0	1	0	0
iMODE 52	CKD_BIO_2103	M	2	72	No	1	1,3	10	IgA nephropath		27/06/2014	3,73	15,20	4	- 6,47	17,46	-42,6	Progressor	23/10/2015	5,11	10,30	5	1	1	1	1
iMODE 47	CKD_BIO_2105	M	2	19	No	1	1	3	Mesangiocapill		14/08/2014	1,20	87,12	2	- 26,77	92,41	-30,7	Progressor	21/10/2015	1,73	55,44	3	1	0	0	1
iMODE 42	CKD_BIO_2111	F	1	69	No	1	0,5	2	Mesangial proli		11/03/2015	1,06	53,50	3	- 48,28	53,50	-90,2	Progressor	24/08/2015	1,74	29,36	4	1	0	0	0
iMODE 44	CKD_BIO_2305	M	2	28	No	1	0,5	2	Focal segmenta		27/04/2015	2,19	39,40	3	- 17,77	39,40	-45,1	Progressor	13/10/2015	2,70	30,52	3	1	0	0	0
iMODE 70	CKD_BIO_2309	M	2	75	No	1			Focal segmenta	dialysis	27/04/2015	3,57	15,70	4				Progressor					0	0	0	0
iMODE 43	CKD_BIO_2310	M	2	54	No	1	0,5	2	Mesangial proli		27/04/2015	1,82	41,02	3	- 37,68	41,02	-91,8	Progressor	10/10/2015	3,02	22,18	4	1	0	0	0
iMODE 11	UZ_GENT_0022	M	2	79	Yes	2	3,1	16	Diabetic nephr		27/01/2011	2,78	20,56	4	- 0,93	22,40	-4,5	Progressor	07/03/2014	3,09	17,71	4	1	1	0	1
iMODE 34	UZ_GENT_0062	F	1	53	No	1	2,5	4	IgA nephropath		28/02/2011	0,84	79,61	2	1,84	76,03	2,3	Non-progresso	22/08/2013	0,80	82,68	2	1	0	0	0
iMODE 13	UZ_GENT_0120	M	2	82	No	1	2,9	9	Chronic hyperte		31/03/2011	1,42	45,67	3	- 2,16	47,61	-4,7	Progressor	25/02/2014	1,44	43,97	3	0	1	0	0
iMODE 31	UZ_GENT_0122	M	2	79	Yes	2	2,8	17	Granulomatosis		23/03/2011	1,45	45,48	3	0,62	44,37	1,4	Non-progresso	07/01/2014	1,53	41,73	3	1	0	0	0
iMODE 36	UZ_GENT_0149	F	1	67	No	1	3,1	11	Primary focal se		19/04/2011	1,06	54,46	3	1,43	51,86	2,6	Non-progresso	22/05/2014	1,03	55,21	3	1	1	0	1
iMODE 12	UZ_GENT_0173	M	2	68	No	1	2,9	19	Chronic hyperte		29/04/2011	1,64	42,34	3	- 1,64	40,57	-3,9	Progressor	03/04/2014	1,82	36,55	3	1	1	0	0
iMODE 15	UZ_GENT_0177	M	2	35	Yes	2	3	10	Adult nephrotic		03/05/2011	1,13	83,74	2	- 2,94	78,70	-3,5	Progressor	08/05/2014	1,27	71,20	2	1	0	0	0
iMODE 21	UZ_GENT_0218	F	1	56	No	1	3	7	Systemic lupus		26/05/2011	0,71	95,52	1	- 1,74	85,80	-1,8	Non-progresso	05/06/2014	0,75	87,53	2	0	1	0	1
iMODE 7	UZ_GENT_0226	F	1	70	No	1	3	27	Systemic vascu		06/06/2011	3,13	14,40	5	- 1,14	14,47	-7,9	Progressor	02/06/2014	4,15	10,03	5	0	1	0	1
iMODE 39	UZ_GENT_0321	M	2	72	No	1	2,7	12	Chronic hyperte		30/08/2011	1,90	34,45	3	1,32	35,96	3,8	Non-progresso	21/05/2014	1,95	32,69	3	0	0	0	0
iMODE 30	UZ_GENT_0329	M	2	50	Yes	2	2,5	5	Acute kidney in		07/09/2011	1,23	68,02	2	0,62	74,08	0,9	Non-progresso	20/02/2014	1,14	73,01	2	1	1	0	0
iMODE 28	UZ_GENT_0333	M	2	54	No	1	2,8	11	IgA nephropath		08/09/2011	1,40	56,56	3	2,44	53,85	4,3	Non-progresso	12/06/2014	1,32	59,88	3	1	0	0	1
iMODE 27	UZ_GENT_0385	M	2	67	No	1	2,5	21	Chronic hyperte		14/10/2011	1,52	46,74	3	- 0,22	43,00	-0,5	Non-progresso	10/04/2014	1,86	35,85	3	1	1	1	0
iMODE 4	UZ_GENT_0386	M	2	76	Yes	2	2,1	14	Diabetic nephr		14/10/2011	1,03	70,23	2	- 2,97	58,68	-4,2	Progressor	03/12/2013	1,13	61,91	2	0	1	1	1
iMODE 3	UZ_GENT_0415	F	1	42	No	1	2,3	16	Systemic lupus		01/12/2011	1,66	37,74	3	- 4,21	41,00	-11,2	Progressor	18/03/2014	1,65	37,23	3	1	0	0	1
iMODE 33	UZ_GENT_0427	M	2	25	No	1	2,5	16	Haemolytic ura		14/12/2011	1,26	78,76	2	2,18	81,88	2,8	Non-progresso	16/06/2014	1,22	80,75	2	1	0	0	0
iMODE 38	UZ_GENT_0437	M	2	72	Yes	2	2,4	9	Diabetic nephr		22/12/2011	1,84	35,82	3	0,42	34,47	1,2	Non-progresso	23/05/2014	1,90	33,73	3	1	0	0	0
iMODE 5	UZ_GENT_0448	M	2	68	Yes	2	2,1	18	Diabetic nephr		10/01/2012	1,07	70,95	2	- 3,36	64,25	-4,7	Progressor	04/02/2014	1,24	58,53	3	1	1	0	0
iMODE 1	UZ_GENT_0458	F	1	73	Yes	2	2,1	8	Diabetic nephr		26/01/2012	2,59	17,73	4	- 4,41	22,21	-24,9	Progressor	20/02/2014	3,30	13,04	5	1	1	0	1
iMODE 22	UZ_GENT_0463	M	2	53	No	1	2	3	Acute kidney in		03/02/2012	1,01	84,52	2	- 1,57	82,39	-1,9	Non-progresso	03/02/2014	1,03	81,39	2	1	1	0	0
iMODE 6	UZ_GENT_0478	M	2	67	No	1	2,1	13	Acute kidney in		24/02/2012	1,96	34,37	3	- 1,59	29,19	-4,6	Progressor	14/03/2014	2,74	22,44	4	0	1	1	0
iMODE 25	UZ_GENT_0506	F	1	80	Yes	2	2,2	27	Diabetic nephr		23/03/2012	1,79	26,38	4	3,59	18,04	13,6	Non-progresso	10/06/2014	1,82	25,50	4	0	0	0	1
iMODE 35	UZ_GENT_0533	M	2	79	No	1	2	5	Henoch-Schönl		14/05/2012	1,35	49,58	3	1,14	49,36	2,3	Non-progresso	19/05/2014	1,28	52,14	3	1	0	0	1
iMODE 2	UZ_GENT_0543	M	2	62	No	1	2	9	IgA nephropath		01/06/2012	3,91	15,45	4	- 1,97	15,22	-12,8	Progressor	23/05/2014	4,92	11,54	5	1	0	0	0

Supplementary Table 2: The 24 miRNAs with adjusted p-value < 0.05 and the miR-30a-5p which was selected as normalizer

miRNA	mean coverage	A=Non Progressor	B=Progressors	BaseMeanB / baseMeanA	2log (baseMeanB / BaseMeanA)	unadjusted p-value	adjusted p-value	rank	sample detected / 70	number non progressors / 35	number progressors /35
hsa-miR-34c-5p	1,125	0,231	2,019	8,743	3,128	4,56E-21	3,16E-18	1	26	8	18
hsa-miR-3200-5p	0,222	0,012	0,431	35,233	5,139	2,87E-09	9,93E-07	2	14	2	12
hsa-miR-944	1,408	0,280	2,536	9,068	3,181	2,26E-08	5,23E-06	3	19	7	12
hsa-miR-34b-3p	0,117	0,006	0,228	39,715	5,312	1,17E-06	2,03E-04	4	9	1	8
hsa-miR-147b	0,425	0,492	0,358	0,727	-0,459	5,08E-06	5,86E-04	5	18	11	7
hsa-miR-410-3p	0,672	0,492	0,853	1,734	0,794	4,64E-06	5,86E-04	6	26	11	15
hsa-miR-513c-5p	0,140	0,000	0,281	Inf	Inf	6,69E-06	6,62E-04	7	5	0	5
hsa-miR-3928-3p	0,192	0,023	0,360	15,575	3,961	1,69E-05	1,46E-03	8	11	3	8
hsa-miR-1289	0,172	0,345	0,000	0,000	#NAME?	2,69E-05	1,87E-03	9	3	3	0
hsa-miR-1323	0,049	0,097	0,000	0,000	#NAME?	2,69E-05	1,87E-03	10	3	3	0
hsa-miR-324-5p	1,061	1,631	0,491	0,301	-1,732	3,93E-05	2,48E-03	11	29	14	15
hsa-miR-3150a-3p	0,168	0,008	0,327	38,655	5,273	7,16E-05		12	7	1	6
hsa-miR-892c-3p	0,088	0,000	0,177	Inf	Inf	1,04E-04	5,53E-03	13	3	0	3
hsa-miR-369-3p	0,255	0,098	0,413	4,228	2,080	1,19E-04	5,89E-03	14	8	1	7
hsa-miR-301b-3p	0,838	0,383	1,293	3,377	1,756	3,81E-04	1,76E-02	15	23	6	17
hsa-miR-449b-5p	0,228	0,095	0,362	3,815	1,932	4,06E-04	1,76E-02	16	9	3	6
hsa-miR-651-5p	0,295	0,076	0,513	6,723	2,749	5,48E-04	2,23E-02	17	14	4	10
hsa-miR-561-5p	0,866	0,788	0,944	1,198	0,260	7,27E-04	2,67E-02	18	33	12	21
hsa-miR-708-5p	0,333	0,368	0,298	0,809	-0,306	7,32E-04	2,67E-02	19	16	7	9
hsa-miR-323b-3p	0,156	0,182	0,130	0,717	-0,480	8,96E-04	2,96E-02	20	11	6	5
hsa-miR-493-5p	0,055	0,007	0,103	15,238	3,930	8,91E-04	2,96E-02	21	6	1	5
hsa-miR-487b-3p	0,324	0,125	0,523	4,172	2,061	1,15E-03	3,62E-02	22	17	6	11
hsa-miR-145-5p	0,246	0,084	0,408	4,850	2,278	1,39E-03	4,18E-02	23	10	3	7
hsa-miR-508-3p	1,190	0,561	1,820	3,241	1,696	1,56E-03	4,50E-02	24	34	13	21
hsa-miR-30a-5p	15854,12061	16008,5163	15699,72491	0,980710805	-0,028100322	9,04E-01	1		70	35	35

Supplementary Table 3: The 142 significantly different urinary proteins (corrected p-value < 0.05, ratio ≤0.66 or ≥ 1.5)

Symbol	Name	Peptide occurences	p-value Mann-Whitney U-test	Benjamini-Hochberg corrected p-value Mann-Whitney U-test	ratio progressors/non-progressors
TF	Serotransferrin	65	1,36E-07	4,69E-05	5,71
HPX	Hemopexin	19	1,48E-06	2,55E-04	8,30
CP	Ceruloplasmin	31	2,52E-06	2,89E-04	3,13
SERPINA1	Alpha-1-antitrypsin	21	6,64E-06	4,57E-04	6,28
GC	Vitamin D-binding protein	11	1,68E-05	5,26E-04	5,50
ORM2	Alpha-1-acid glycoprotein 2	11	2,14E-05	6,14E-04	2,84
IGHG1	Ig gamma-1 chain C region	11	3,86E-05	1,02E-03	3,79
A1BG	Alpha-1B-glycoprotein	11	5,46E-05	1,17E-03	3,15
AFM	Afamin	12	8,83E-05	1,42E-03	3,68
ALB	Serum albumin	80	1,33E-04	1,64E-03	2,86
IGHG2	Ig gamma-2 chain C region	18	1,75E-04	1,72E-03	2,09
UBC	Polyubiquitin-C	3	2,22E-04	1,96E-03	1,52
ORM1	Alpha-1-acid glycoprotein 1	7	4,69E-04	3,21E-03	2,86
CD320	CD320 antigen	2	5,36E-04	3,36E-03	1,71
Ig heavy chain V-III region BRO	Ig heavy chain V-III region BRO	2	1,50E-03	6,88E-03	2,48
IGHA1	Ig alpha-1 chain C region	15	1,92E-03	8,49E-03	2,54
LRG1	Leucine-rich alpha-2-glycoprotein	13	2,15E-03	8,93E-03	2,80
AZGP1	Zinc-alpha-2-glycoprotein	26	3,65E-03	1,23E-02	2,00
APOA1	Apolipoprotein A-I	5	4,32E-03	1,35E-02	7,55
NGFR	Tumor necrosis factor receptor superfamily member 16	3	4,77E-03	1,46E-02	1,63
RBP4	Retinol-binding protein 4	6	5,56E-03	1,58E-02	4,14
SERPINA3	Alpha-1-antichymotrypsin	17	6,55E-03	1,76E-02	2,60
TFF2	Trefoil factor 2	1	7,35E-03	1,89E-02	1,60
IGKC	Ig kappa chain C region	7	7,69E-03	1,90E-02	2,14
TTR	Transthyretin	1	9,43E-03	2,16E-02	11,95
AHSG	Alpha-2-HS-glycoprotein	10	9,75E-03	2,19E-02	5,21
HP	Haptoglobin	13	1,05E-02	2,32E-02	3,82
FBLN5	Fibulin-5	2	1,49E-02	3,12E-02	1,69
AGT	Angiotensinogen	4	1,54E-02	3,18E-02	5,09
SERPINC1	Antithrombin-III	3	1,59E-02	3,27E-02	5,80
A2M	Alpha-2-macroglobulin	1	2,24E-02	4,10E-02	5,71
VTN	Vitronectin	7	2,54E-02	4,41E-02	2,58
SERPINA7	Thyroxine-binding globulin	5	2,54E-02	4,41E-02	1,92
KRT10	Keratin, type I cytoskeletal 10	7	2,93E-02	4,92E-02	3,18
IGSF8	Immunoglobulin superfamily member 8	4	6,56E-06	4,57E-04	0,27
PGLYRP1	Peptidoglycan recognition protein 1	4	1,24E-05	4,73E-04	0,65
GP6	Platelet glycoprotein VI	2	1,16E-05	4,73E-04	0,35
MXRA8	Matrix-remodeling-associated protein 8	4	1,37E-05	4,73E-04	0,32
DEFB1	Beta-defensin 1	1	9,16E-06	4,73E-04	0,21
RELT	Tumor necrosis factor receptor superfamily member 19L	1	1,03E-05	4,73E-04	0,13
COL15A1	Collagen alpha-1(XV) chain	3	4,70E-05	1,15E-03	0,13

Symbol	Name	Peptide occurences	p-value Mann-Whitney U-test	Benjamini-Hochberg corrected p-value Mann-Whitney U-test	ratio progressors/non-progressors
TWSG1	Twisted gastrulation protein homolog 1	2	5,68E-05	1,17E-03	0,63
NEGR1	Neuronal growth regulator 1	3	5,93E-05	1,17E-03	0,44
PVRL1	Nectin-1	1	6,12E-05	1,17E-03	0,18
ACAN	Aggrecan core protein	2	7,00E-05	1,27E-03	0,28
RETN	Resistin	2	9,34E-05	1,42E-03	0,51
CNTFR	Ciliary neurotrophic factor receptor subunit alpha	2	9,49E-05	1,42E-03	0,47
NEU1	Sialidase-1	3	9,38E-05	1,42E-03	0,37
AMY2A	Pancreatic alpha-amylase	5	1,11E-04	1,49E-03	0,48
CPN2	Carboxypeptidase N subunit 2	4	1,11E-04	1,49E-03	0,34
LYNX1	Ly-6/neurotoxin-like protein 1	1	1,12E-04	1,49E-03	0,33
QPCT	Glutaminyl-peptide cyclotransferase	7	1,57E-04	1,64E-03	0,36
CADM1	Cell adhesion molecule 1	2	1,57E-04	1,64E-03	0,34
PRCP	Lysosomal Pro-X carboxypeptidase	2	1,43E-04	1,64E-03	0,34
AMY2B	Alpha-amylase 2B	1	1,49E-04	1,64E-03	0,21
ASGR2	Asialoglycoprotein receptor 2	1	1,75E-04	1,72E-03	0,14
TINAGL1	Tubulointerstitial nephritis antigen-like	1	1,94E-04	1,86E-03	0,18
SERPINA5	Plasma serine protease inhibitor	9	2,05E-04	1,91E-03	0,36
SIRPB1	Signal-regulatory protein beta-1	4	2,14E-04	1,94E-03	0,58
ISLR	Immunoglobulin superfamily containing leucine-rich repeat protein	1	2,28E-04	1,96E-03	0,21
CLEC3B	Tetranectin	2	2,78E-04	2,28E-03	0,24
CLEC4G	C-type lectin domain family 4 member G	1	2,74E-04	2,28E-03	0,18
MUC1	Mucin-1	3	3,09E-04	2,47E-03	0,32
TNFRSF1B	Tumor necrosis factor receptor superfamily member 1B	1	3,41E-04	2,50E-03	0,63
VMO1	Vitelline membrane outer layer protein 1 homolog	2	3,41E-04	2,50E-03	0,37
PEBP1	Phosphatidylethanolamine-binding protein 1	2	3,33E-04	2,50E-03	0,31
APOD	Apolipoprotein D	15	4,46E-04	3,20E-03	0,43
EGF	Pro-epidermal growth factor	19	4,59E-04	3,21E-03	0,36
CPM	Carboxypeptidase M	1	4,93E-04	3,26E-03	0,31
CD9	CD9 antigen	1	5,38E-04	3,36E-03	0,65
MCAM	Cell surface glycoprotein MUC18	1	5,73E-04	3,52E-03	0,27
NID1	Nidogen-1	3	5,86E-04	3,53E-03	0,55
PVRL2	Nectin-2	3	6,57E-04	3,83E-03	0,56
CLN5	Ceroid-lipofuscinosis neuronal protein 5	1	7,41E-04	4,18E-03	0,42
HYAL1	Hyaluronidase-1	1	7,33E-04	4,18E-03	0,27
TNFRSF14	Tumor necrosis factor receptor superfamily member 14	2	7,63E-04	4,23E-03	0,45
SDC4	Syndecan-4	1	8,27E-04	4,52E-03	0,27
PGLS	6-phosphogluconolactonase	5	9,71E-04	5,22E-03	0,60
SPINT1	Kunitz-type protease inhibitor 1	1	1,01E-03	5,36E-03	0,31
SPINT2	Kunitz-type protease inhibitor 2	1	1,08E-03	5,62E-03	0,44
BHMT	Betaine--homocysteine S-methyltransferase 1	2	1,16E-03	5,91E-03	0,41
CDH2	Cadherin-2	1	1,24E-03	6,10E-03	0,37
GGT3P	Putative gamma-glutamyltranspeptidase 3	1	1,30E-03	6,30E-03	0,22
CSPG4	Chondroitin sulfate proteoglycan 4	4	1,34E-03	6,42E-03	0,64
EIF6	Eukaryotic translation initiation factor 6	2	1,50E-03	6,88E-03	0,49

Symbol	Name	Peptide occurences	p-value Mann-Whitney U-test	Benjamini-Hochberg corrected p-value Mann-Whitney U-test	ratio progressors/non-progressors
SCPEP1	Retinoid-inducible serine carboxypeptidase	1	1,49E-03	6,88E-03	0,23
CD84	SLAM family member 5	2	1,79E-03	7,99E-03	0,55
HEXA	Beta-hexosaminidase subunit alpha	2	2,05E-03	8,85E-03	0,47
PLD3	Phospholipase D3	1	2,06E-03	8,85E-03	0,22
PRSS8	Prostasin	1	2,14E-03	8,93E-03	0,44
ACP2	Lysosomal acid phosphatase	7	2,19E-03	8,98E-03	0,56
PROCR	Endothelial protein C receptor	4	2,26E-03	9,15E-03	0,47
ABHD14B	Alpha/beta hydrolase domain-containing protein 14B	1	2,31E-03	9,22E-03	0,33
AXL	Tyrosine-protein kinase receptor UFO	5	2,36E-03	9,28E-03	0,59
CEL	Bile salt-activated lipase	2	2,37E-03	9,28E-03	0,37
KLK1	Kallikrein-1	2	2,42E-03	9,35E-03	0,52
LDLR	Low-density lipoprotein receptor	1	2,47E-03	9,44E-03	0,60
NUCB1	Nucleobindin-1	1	2,63E-03	9,84E-03	0,58
ALDOB	Fructose-bisphosphate aldolase B	1	2,69E-03	9,95E-03	0,24
OSCAR	Osteoclast-associated immunoglobulin-like receptor	4	3,12E-03	1,13E-02	0,44
COL6A1	Collagen alpha-1(VI) chain	9	3,20E-03	1,15E-02	0,49
HEG1	Protein HEG homolog 1	3	3,28E-03	1,16E-02	0,61
ZG16B	Zymogen granule protein 16 homolog B	1	3,35E-03	1,17E-02	0,33
PCOLCE	Procollagen C-endopeptidase enhancer 1	3	3,40E-03	1,18E-02	0,62
PGA4	Pepsin A-4	3	3,81E-03	1,23E-02	0,57
FGA	Fibrinogen alpha chain	4	3,81E-03	1,23E-02	0,57
HAVCR2	Hepatitis A virus cellular receptor 2	6	3,65E-03	1,23E-02	0,54
ICAM2	Intercellular adhesion molecule 2	1	3,76E-03	1,23E-02	0,32
PROZ	Vitamin K-dependent protein Z	3	3,81E-03	1,23E-02	0,27
PTPRG	Receptor-type tyrosine-protein phosphatase gamma	1	4,14E-03	1,31E-02	0,48
COMP	Cartilage oligomeric matrix protein	1	4,52E-03	1,40E-02	0,37
APOE	Apolipoprotein E	2	4,87E-03	1,48E-02	0,53
COL18A1	Collagen alpha-1(XVIII) chain	1	4,91E-03	1,48E-02	0,36
PEBP4	Phosphatidylethanolamine-binding protein 4	1	5,31E-03	1,56E-02	0,52
CD248	Endosialin	11	5,56E-03	1,58E-02	0,64
VASN	Vasorin	6	5,56E-03	1,58E-02	0,61
THBD	Thrombomodulin	1	5,93E-03	1,67E-02	0,64
OLFM4	Olfactomedin-4	4	6,52E-03	1,76E-02	0,59
CD300A	CMRF35-like molecule 8	3	6,41E-03	1,76E-02	0,56
NHLRC3	NHL repeat-containing protein 3	3	6,41E-03	1,76E-02	0,50
PDGFRB	Platelet-derived growth factor receptor beta	1	6,68E-03	1,78E-02	0,52
FZD2	Frizzled-2	1	6,79E-03	1,80E-02	0,63
PVR	Poliovirus receptor	3	7,00E-03	1,82E-02	0,44
LDHB	L-lactate dehydrogenase B chain	3	7,28E-03	1,88E-02	0,57
MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	1	7,68E-03	1,90E-02	0,49
ACVR1B	Activin receptor type-1B	1	7,58E-03	1,90E-02	0,44
EPHB6	Ephrin type-B receptor 6	1	7,69E-03	1,90E-02	0,43
EPHB4	Ephrin type-B receptor 4	4	7,91E-03	1,92E-02	0,63
IL18BP	Interleukin-18-binding protein	1	7,88E-03	1,92E-02	0,55

Symbol	Name	Peptide occurences	p-value Mann-Whitney U-test	Benjamini-Hochberg corrected p-value Mann-Whitney U-test	ratio progressors/non-progressors
GLB1	Beta-galactosidase	4	1,01E-02	2,26E-02	0,62
IL6ST	Interleukin-6 receptor subunit beta	1	1,05E-02	2,32E-02	0,32
MASP2	Mannan-binding lectin serine protease 2	6	1,32E-02	2,83E-02	0,62
PSCA	Prostate stem cell antigen	1	1,39E-02	2,95E-02	0,48
SERPINF2	Alpha-2-antiplasmin	1	1,40E-02	2,96E-02	0,61
GRN	Granulins	4	1,65E-02	3,31E-02	0,45
FOLR1	Folate receptor alpha	5	1,71E-02	3,37E-02	0,55
IGFBP7	Insulin-like growth factor-binding protein 7	11	2,13E-02	3,94E-02	0,61
COTL1	Coactosin-like protein	1	2,33E-02	4,17E-02	0,59
NUTF2	Nuclear transport factor 2	2	2,55E-02	4,41E-02	0,64
ACPP	Prostatic acid phosphatase	7	2,72E-02	4,66E-02	0,40
LCN2	Neutrophil gelatinase-associated lipocalin	1	2,81E-02	4,79E-02	0,38
Ig kappa chain V-I region Roy	Ig kappa chain V-I region Roy	1	2,91E-02	4,90E-02	0,63

Supplementary Table 4: The 7 miRNAs with the 15 predicted targets and molecular pathways involved. In bold the 4 miRNAs that were further validated.

Symbol	Exp Fold Change	Confidence	ID	Symbol	Exp Fold Change	Pathway
miR-301b-3p	3.377	High (predicted)	P54760	EPHB4	-1.586	Axonal Guidance Signaling,Ephrin B Signaling,Ephrin Receptor Signaling
		High (predicted)	P40189	IL6ST	-3.173	Acute Phase Response Signaling,CNTF Signaling, ERK5 Signaling,IL-6 Signaling,Role of JAK family kinases in IL-6-type Cytokine Signaling,Role of Macrophages, T Helper Cell Differentiation
		High (predicted)	P01130	LDLR	-1.671	Clathrin-mediated Endocytosis Signaling,LXR/RXR Activation,TR/RXR Activation
		High (predicted)	P23470	PTPRG	-2.078	Protein Kinase A Signaling
		High (predicted)	P20333	TNFRSF1B	-1.587	Acute Phase Response Signaling,Apoptosis Signaling, Crosstalk between Dendritic Cells and Natural Killer Cells,Death Receptor Signaling,Dendritic Cell Maturation, IL-6 Signaling,LPS/IL-1 Mediated Inhibition of RXR Function,LXR/RXR Activation,NF-κB Signaling,p38 MAPK Signaling,PPAR Signaling,Production of Nitric Oxide and Reactive Oxygen Species in Macrophages,Role of Macrophages, T Helper Cell Differentiation,Tight Junction Signaling,TNFR2 Signaling,Type I Diabetes Mellitus Signaling,Type II Diabetes Mellitus Signaling
miR-145-5p	4.850	High (predicted)	P36896	ACVR1B	-2.278	Epithelial Adherens Junction Signaling, PPARα/RXRα Activation,TGF-β Signaling,Wnt/β-catenin Signaling
		High (predicted)	P19022	CDH2	-2.738	Epithelial Adherens Junction Signaling,Germ Cell-Sertoli Cell Junction Signaling,Gα12/13 Signaling,Regulation of the Epithelial-Mesenchymal Transition Pathway,RhoGDI Signaling,Signaling by Rho Family GTPases,Wnt/β-catenin Signaling
		High (predicted)	P01133	EGF	-2.788	Actin Cytoskeleton Signaling,EGF Signaling,Ephrin Receptor Signaling,Epithelial Adherens Junction Signaling,ErbB Signaling,ERK5 Signaling,FAK Signaling,Gap Junction Signaling, Signaling,IL-8 Signaling,,,NF-κB Signaling,Regulation of Cellular Mechanics by Calpain Protease,Regulation of the Epithelial-Mesenchymal Transition Pathway,Telomerase Signaling,Thrombin Signaling
		Experimentally Observed	P15941	MUC1	-3.156	ILK Signaling
		High (predicted)	Q15223	NECTIN1	-5.588	Epithelial Adherens Junction Signaling,Sertoli Cell-Sertoli Cell Junction Signaling,Tight Junction Signaling

Symbol	Exp Fold Change	Confidence	ID	Symbol	Exp Fold Change	Pathway
miR-410-3p	1.734	High (predicted)	Q14332	FZD2	-1.584	Regulation of the Epithelial-Mesenchymal Transition Pathway,Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis, Wnt/Ca+ pathway,Wnt/β-catenin Signaling
		High (predicted)	P01130	LDLR	-1.671	Clathrin-mediated Endocytosis Signaling,LXR/RXR Activation,TR/RXR Activation
miR-34c-5p	8.743	High (predicted)	Q15223	NECTIN1	-5.588	Epithelial Adherens Junction Signaling,Sertoli Cell-Sertoli Cell Junction Signaling,Tight Junction Signaling
		High (predicted)	P09619	PDGFRB	-1.921	3-phosphoinositide Biosynthesis, NF-κB Signaling,PAK Signaling,PDGF Signaling,PPAR Signaling,PTEN Signaling,Regulation of the Epithelial-Mesenchymal Transition Pathway,Sphingosine-1-phosphate Signaling,STAT3 Pathway,Superpathway of Inositol Phosphate Compounds
		High (predicted)	P08697	SERPINF2	-1.640	Acute Phase Response Signaling,Coagulation System,FXR/RXR Activation,LXR/RXR Activation
miR-3150a-3p	38.655	High (predicted)	Q15223	NECTIN1	-5.588	Epithelial Adherens Junction Signaling,Sertoli Cell-Sertoli Cell Junction Signaling,Tight Junction Signaling
miR-3928-3p	15.575	High (predicted)	P02790	HPX	8.296	Acute Phase Response Signaling,FXR/RXR Activation,LXR/RXR Activation
miR-708-5p	-1.237	High (predicted)	P26992	CNTFR	-2.110	CNTF Signaling

Supplementary Table 5: Disease and functions the selected miRNAs and their targets are predicted to be involved.

Categories	Diseases or Functions Annotation	p-Value	Predicted Activation State	Molecules
Cell Death and Survival	apoptosis	4,64E-06	Increased	IL6ST,EPHB4,CDH2,miR-34c-5p,LDLR,miR-145-5p,CNTFR,MUC1,EGF,TNFRSF1B,FZD2,ACVR1B,PDGFRB
Cell Death and Survival	cell death	1,26E-05	Increased	IL6ST,EPHB4,MUC1,CNTFR,EGF,SERPINF2,ACVR1B,miR-34c-5p,CDH2,LDLR,miR-145-5p,TNFRSF1B,FZD2,PDGFRB
Renal Necrosis/Cell Death	cell death of kidney cell lines	0,00531	Increased	miR-34c-5p,EGF,TNFRSF1B
Renal Necrosis/Cell Death	apoptosis of kidney cell lines	0,0304	Increased	miR-34c-5p,EGF
Cell Death and Survival	cell survival	3,81E-06	Decreased	IL6ST,EPHB4,miR-34c-5p,CDH2,PTPRG,CNTFR,EGF,TNFRSF1B,FZD2,PDGFRB
Cell Death and Survival	cell viability	0,00002	Decreased	IL6ST,EPHB4,miR-34c-5p,CDH2,PTPRG,EGF,TNFRSF1B,FZD2,PDGFRB
Cellular Growth and Proliferation, Tissue Development	generation of cells	0,00013	Decreased	IL6ST,EPHB4,HPX,miR-34c-5p,CDH2,LDLR,EGF,NECTIN1,TNFRSF1B,PDGFRB
Renal Inflammation, Renal Nephritis	Nephritis	0,0538		MUC1,miR-301b-3p
Renal Inflammation, Renal Nephritis	class II lupus nephritis	0,107		miR-301b-3p

6. SYSTEMS BIOLOGY COMBINING HUMAN- AND ANIMAL-DATA MIRNA AND MRNA DATA IDENTIFIES NEW TARGETS IN URETEROPELVIC JUNCTION OBSTRUCTION

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Papadopoulos et al., BMC systems biology.

6.1. ABSTRACT

Background

Although renal fibrosis and inflammation have shown to be involved in the pathophysiology of obstructive nephropathies, molecular mechanisms underlying evolution of these processes remain undetermined. In an attempt towards improved understanding of obstructive nephropathy and improved translatability of the results to clinical practice we have developed a systems biology approach combining omics data of both human and mouse obstructive nephropathy.

Results

We have studied in parallel the urinary miRNome of infants with ureteropelvic junction obstruction and the kidney tissue miRNome and transcriptome of the corresponding neonatal partial unilateral ureteral obstruction (UUO) mouse model. Several hundreds of miRNAs and mRNAs displayed changed abundance during disease. Combination of miRNAs in both species and associated mRNAs let to the prioritization of 5 miRNAs and 35 mRNAs associated to disease. *In vitro* and *in vivo* validation identified consistent dysregulation of let-7a-5p and miR-29-3p and new potential targets, E3 ubiquitin-protein ligase **(**DTX4) and neuron navigator 1 (NAV1), potentially involved in fibrotic processes, in obstructive nephropathy in both human and mice that would not be identified otherwise.

Conclusions

Our study is the first to correlate a mouse model of neonatal partial UUO with human UPJ obstruction in a comprehensive systems biology analysis. Our data revealed let-7a and miR-29b as molecules potentially involved in the development of fibrosis in UPJ obstruction via the control of DTX4 in both man and mice that would not be identified otherwise.

Keywords

- Obstructive nephropathy
- miRNAs / microRNAs
- microarrays
- let-7a-5p and miR-29b-3p
- DTX4 and NAV1

6.2. Background

Congenital obstructive nephropathy is the main cause of end stage renal disease (ESRD) in children **(Warady and Chadha, 2007)**. This contrasts sharply with adult ESRD, which for the greater part originates from type II diabetes and hypertension **(Chevalier, 2015a)**. The most frequent cause of congenital urinary tract obstruction is ureteropelvic junction (UPJ) obstruction with an estimated incidence of 1 in 1000–1500 **(Chang et al., 2004)**. The spectrum of renal abnormalities varies greatly in UPJ obstruction ranging from subtle changes such as modified proximal or tubular size, chronic tubulointerstitial injury, glomerulosclerosis, fibrosis, aberration of nephron development and in severe cases (less than 1%) renal dysplasia **(Klein et al., 2011)**. The gold standard in diagnosis of UPJ is by prenatal ultrasonography with subsequent evaluation in the postnatal period **(Chevalier, 2015b)**. However, this method is not sensitive enough to accurately estimate renal function and functioning nephron number **(Waikar et al., 2009)**. This has led to an urgent need for the development of biomarkers to assess the severity of UPJ obstruction and to help the clinicians to decide if and when pyeloplasty is required **(Chevalier, 2015b, Chertin et al., 2006)**.

Due to the fact that limited human kidney samples are available, almost all observations on the pathophysiology of UPJ obstruction are based on animal models, which potentially limit the transferability of the observations in the clinical context. As a consequence, the pathophysiological mechanisms of UPJ obstruction remain incompletely understood. Renal lesions in UPJ obstruction have been described including tubular proliferation/apoptosis, renin-angiotensin system activation, inflammation, and fibrosis **(Klein et al., 2011, Chevalier, 2015b, Chevalier et al., 2014, Meiri et al., 2012)**. Interstitial fibrosis is a late consequence of congenital UPJ obstruction, and can be attenuated by early release of obstruction, but not if nephron number is reduced **(Sergio et al., 2015)**. It is becoming increasingly clear that patients with congenital urinary tract obstruction must be followed into adulthood, as the lesions can progress over the entire life **(Chevalier, 2015a)**.

MicroRNAs (miRNAs) are small non-coding RNAs (20 - 24 nt) that regulate gene expression by blocking the translation of proteins and are involved in multiple molecular pathways and pathologies. MiRNAs are now considered promising molecules for biomarkers and/or targeted therapy of disease **(Hammond, 2015, Almeida et al., 2011, van Rooij and Kauppinen, 2014, Esteller, 2011)**. While, kidney diseases are no exception **(Schena et al., 2014, Papadopoulos et al., 2015b)**, to our knowledge no studies specifically report miRNAs related to UPJ obstruction. There is evidence connecting dysregulated miRNAs including miR-21 and miR-29 with kidney fibrosis **(Zarjou et al., 2011, Chau et al., 2012, Glowacki et al., 2013, He et al., 2013, Wang et al., 2012a, Qin et al., 2011)**, an important feature in severe UPJ obstruction. Moreover, knock-down of DICER (the main protein involved in the biogenesis of miRNAs **(Bartel, 2004, Denzler and Stoffel, 2015)**) leads to congenital anomalies of the kidney and the urinary tract (CAKUT) in mice **(Bartram et al., 2013)**. MiRNAs are very stable in urine, a biofluid which can be collected non-invasively and can be valuable source of molecules to monitor diseases of the kidney and urinary tract **(Papadopoulos et al., 2015b)**.

These observations suggest that studying miRNAs in UPJ obstruction might help to understand the development of obstructive nephropathy and/or provide early markers of pathological obstruction.

In this study we analysed the modification of urinary miRNAs in UPJ obstruction. To improve upon the clinical translatability of our results and compensate for the lack of tissue availability in human disease, we combined miRNA data obtained in human urine and miRNA and mRNA data in kidney tissue of a neonatal partial unilateral ureteral obstruction (UUO) mouse model. This combined systems biology-based approach followed by an *in vitro* validation pointed to the consistent dysregulation of specific miRNAs, let-7a-5p and miR-29-3p and to new potential targets, E3 ubiquitin-protein ligase **(**DTX4) and neuron navigator 1 (NAV1) in UPJ obstruction that would not be identified otherwise.

6.3. Results

MiRNA abundance changes in urine of newborns with UPJ obstruction

A total of 20 male UPJ obstruction patients and 8 male healthy age-matched (< 1 year-old) individuals were studied (**Table 1**). The severity of the obstruction varied with hydronephrosis grades from 1-4 and pelvic dilatation sizes from 6 to 40 mm (**Table 1**). We compared the urinary miRNA abundance of UPJ obstruction patients to urine of healthy controls using microarray analysis. This yielded the identification of 227 miRNAs with changed urinary abundance between the two groups (unadjusted $p<0.05$, **Additional file 1**).

Table 1: Clinical data of the human UPJ obstruction patients.

	N	**Age (days) at urine sampling *median* [range]**	**HN grade *median* [range]**	**Pelvic diameter mm *median* [range]**
Healthy controls	8	112 [20-201]	n.a.	n.a.
UPJ obstruction	20	74 [3-269]	2 [1-4]	15 [6-40]

HN: hydronephrosis; n.a.: not applicable

MiRNA and mRNA expression changes in renal tissue of a neonatal partial UUO mouse model

The renal miRNA and mRNA profiles of 9 neonatal mice with partial UUO (hydronephrosis grades 2 and 3) and 9 control sham operated mice were studied using microarray analysis (**Table 2**). This led to the identification of 79 differentially expressed miRNAs and 706 differentially expressed mRNAs, respectively (unadjusted $p<0.05$, **Additional file 2 and 3**).

Table 2: Experimental data of the partial UUO model animals.

	N (Male/Female)	**HN grade *median* [range]**	**Pelvic diameter in mm *median* [range]**
Sham	9 (4/5)	n.a.	1.2 [0.8-1.5]
Partial UUO	9 (6/3)	2.5 [2-3]	1.5 [1-2.8]

HN: hydronephrosis; n.a.: not applicable

Identification of most prominent dysregulated miRNAs commonly associated with obstructive nephropathy in humans and mice

To prioritize the molecular features with potentially the highest impact on the development of kidney lesions, we next identified the miRNAs that could consistently reflect the human disease by comparing the similarity of the human (urine) and animal (kidney tissue) miRNA signature taking advantage from the fact that miRNAs are well conserved between species **(Griffiths-Jones et al., 2006)**. The 227 differentially expressed human miRNAs and 79 differentially expressed mouse miRNAs were combined. This led to the identification of 18 common miRNAs **(Additional file 4)** that were reduced to five miRNAs when applying a fold change threshold of 2.5 in mice tissue. These five miRNAs were let-7a-5p miR-16-5p, miR-29b-3p, miR-125b-5p and miR-26a-5p (**Table 3**).

Table 3: Most prominent dysregulated miRNAs commonly associated to the partial UUO model and human UPJ obstruction.

	UPJ vs Healthy (urine)		**Partial UUO vs Sham (kidney)**	
miRNA	**FC**	**p-value**	**FC**	**p-value**
let-7a-5p	-1,559	3,17E-03	-3,558	3,93E-03
miR-16-5p	-1,293	2,27E-03	-2,913	6,97E-04
miR-29b-3p	-1,153	2,93E-02	10,073	3,10E-08
miR-125b-5p	-1,18	3,03E-02	-3,219	3,15E-03
miR-26a-5p	-1,376	3,03E-02	-3,175	1,13E-03

FC: fold change.

Correlation of the urinary abundance of the five miRNAs in UPJ patients with clinical parameters showed that miR-125-5p was inversely correlated with hydronephrosis grade (spearman r = -0,63, p = 0,003, **Table 4**). In addition, a slight but significant inverse correlation with pelvic diameter was also observed for miR-let-7a-5p and miR-125-5p and with hydronephrosis grade for miR-let-7a-5p and miR-26a-5p, and a slight but significant positive correlation with age for miR-let-7a-5p and miR-16-5p (**Table 4**).

Table 4: Correlation of the urinary abundance of the five miRNAs in UPJ patients with clinical parameters

	Pelvic diameter (mm)		Hydronephrosis grade		Age (days)	
	Spearman r	p value	Spearman r	p value	Spearman r	p value
hsa-let-7a-5p	-0,47	0,04	-0,54	0,01	0,53	0,02
hsa-miR-125b-5p	-0,47	0,04	-0,63	0,003	0,26	n.s.
hsa-miR-26a-5p	-0,33	n.s.	-0,50	0,02	0,07	n.s.
hsa-miR-16-5p	-0,20	n.s.	-0,18	n.s.	0,49	0,03
hsa-miR-29b-3p	0,40	n.s.	0,37	n.s.	-0,29	n.s.

Identification of most prominent dysregulated pathways and mRNA targets in obstructive nephropathy

706 differentially expressed mRNAs were observed in kidneys of neonatal mice with partial UUO, including increased expression of markers of fibrosis such as Tgf-β1 and different forms of collagen (**Additional file 3**). To prioritize the mRNA targets, the five selected common miRNAs in mice and human obstructive nephropathy were combined with these 706 differentially expressed mRNAs using Ingenuity Pathway Analysis (IPA).

Then, we focused on the direct connections of the 5 miRNAs as generated from the predicted networks from IPA analysis. This led to the identification of 35 predicted target mRNAs for these 5 miRNAs (**Additional file 5** and **Additional file 6, 7, 8**). Next, in order to focus on the most prominent molecular changes, only mRNAs predicted to be targeted by at least 2 of the 5 miRNAs were kept for further analysis: E3 ubiquitin-protein ligase DXT4 (Dtx4), leiomodin-1 (Lmod1), a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19 (Adamts19) and neuron navigator 1 (Nav1) (**Table 5, Additional file 5**).

Table 5: Most prominent dysregulated mRNA target in obstructive nephropathy.

Gene Symbol Description	FC	p-value	let-7a-5p	miR-125b-5p	miR-16-5p	miR-26a-5p	miR-29b-3p
Dtx4 E3 ubiquitin-protein ligase or deltex 4 homolog	2,41	1,23E-03	√	√			
Lmod1 leiomodin 1 (smooth muscle)	1,57	1,48E-03		√	√		
Adamts19 a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19	2,97	5,67E-04				√	√
Nav1 neuron navigator 1	1,57	1,64E-03				√	√

FC: fold change.

MiRNA knockdown leads to dysregulated expression of the mRNA targets in renal cells *in vitro*

To validate the results of the *in silico* analysis, we next assessed whether the predicted miRNA-mRNA target pairs were directly associated in human renal cells. Human kidney cells (HK2) were treated for 48h with chemically modified molecules blocking the action of specific miRNA (antagomirs). In the presence of antagomirs, the detected signal of let-7a, miR-16-5p, miR-125b-5p, miR-26a-5p and miR-29b-3p was significantly decreased (**Figure 1**).

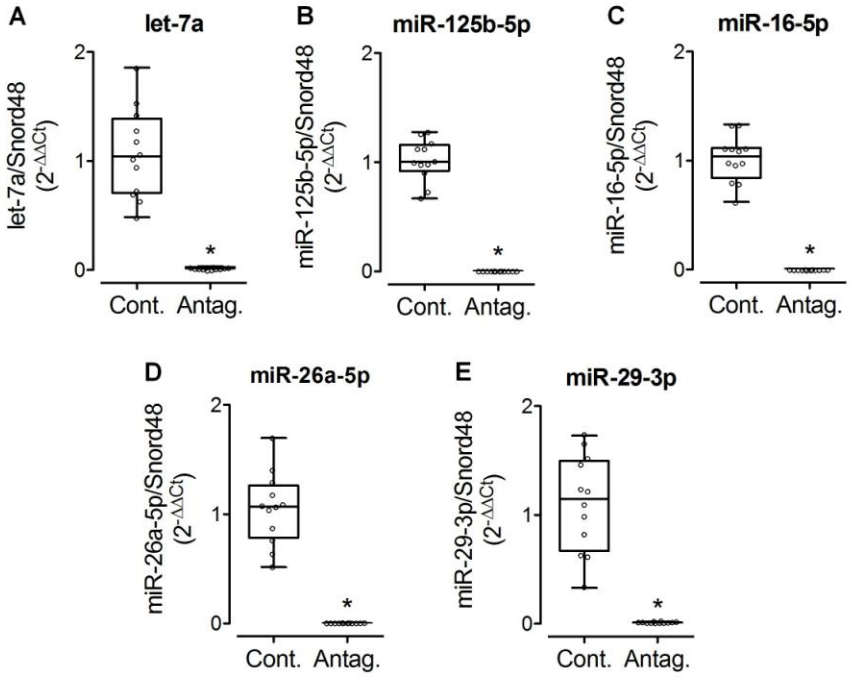

Figure 1. Selected miRNA expression in response to antagomirs *in vitro*. Expression of let-7a (**A**), miR-125b-5p (**B**), miR-16-5p (**C**), miR-26a-5p (**D**) and miR-29b-3p (**E**) was assessed by RT-PCR in HK2 cells treated or not with antagomirs. Cont: control; Antag: antagomir. *p<0.05 versus Cont.

MRNA expression of let-7a and miR-125b-3p target *DTX4* was significantly increased in response to downregulation of let-7a but was unmodified by antagomir anti-miR-125b-3p (**Figure 2A**). Moreover, significant upregulation of neuron navigator 1 *NAV1* was observed in HK2 cells treated with the miR-29b-3p antagomir (**Figure 2D**). Surprisingly, the use of antagomirs for miR-125b-5p and miR-26a-5p resulted in slight but significant downregulation of *LMOD1*, *ADAMTS19* and *NAV1* (**Figures 2B-D**), a result opposite than the predicted regulation, which possibly indicates an indirect mechanism of effect of these two miRNAs on these targets. Antagomirs for miR-125b-5p, miR-16-5p and miR-29b-3p showed no effect on *DTX4*, *LMOD1* and *ADAMTS19*, respectively (**Figure 2A-C**).

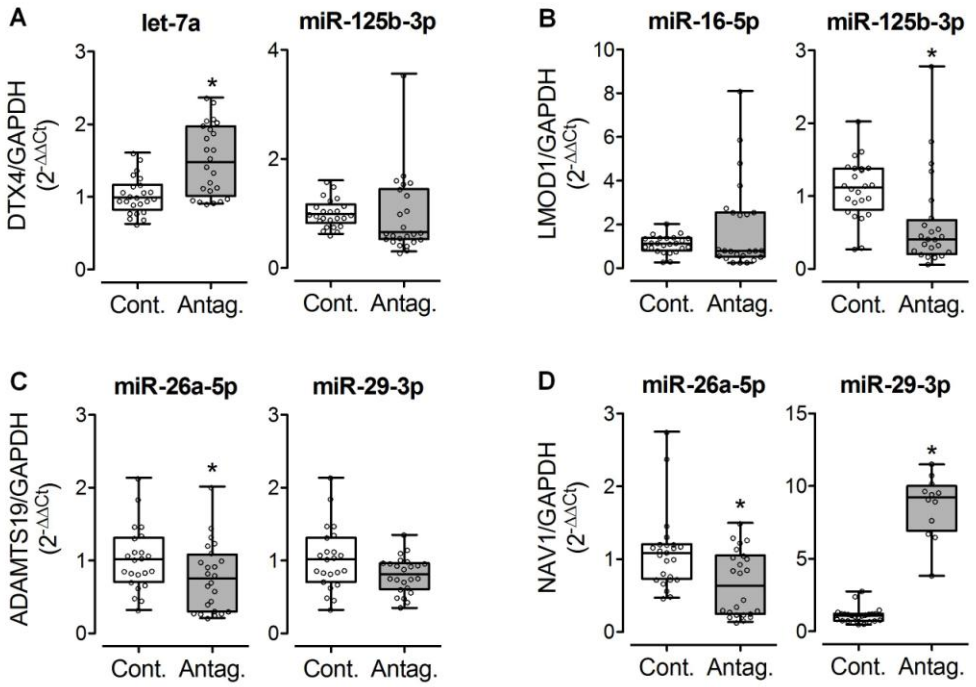

Figure 2. Selected mRNA predicted target expression in response to antagomirs *in vitro*. Expression of E3 ubiquitin-protein ligase DXT4 (DTX4)(**A**), leiomodin-1 (LMOD1)(**B**), a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19 (ADAMTS19)(**C**) and neuron navigator 1 (NAV1)(**D**) was assessed by RT-PCR in HK2 cells treated or not with antagomirs against let-7a, miR-125b-5p, miR-16-5p, miR-26a-5p or miR-29b-3p. Cont: control; Antag: antagomir. *p<0.05 versus Cont.

Dtx4* and *Nav1* are dysregulated during complete UUO *in vivo

Since obstructive nephropathy potentially induces different pathways in the developing and the adult kidney we also verified the expression of the mRNA targets in the adult mouse complete UUO model, using 8 male mice with complete UUO and 6 sham operated mice. We demonstrated that, similarly to what we observed in partial UUO in neonatal mice, *Dtx4* and *Nav1* expression was significantly increased in UUO mice compared to the sham (**Figure 3A and 3C**), while *Lmod1* was not modified (**Figure 3B**). The signal for *Adamts19* was too weak to provide any reliable data. In addition to the targets, we also showed that the renal expression of *Tgf-β* and collagen 1, two markers of fibrosis, and *IL-6*, a marker of inflammation was significantly increased in UUO mice compared to the sham (**Figure 3D-F**), validating the pathological changes observed in this model.

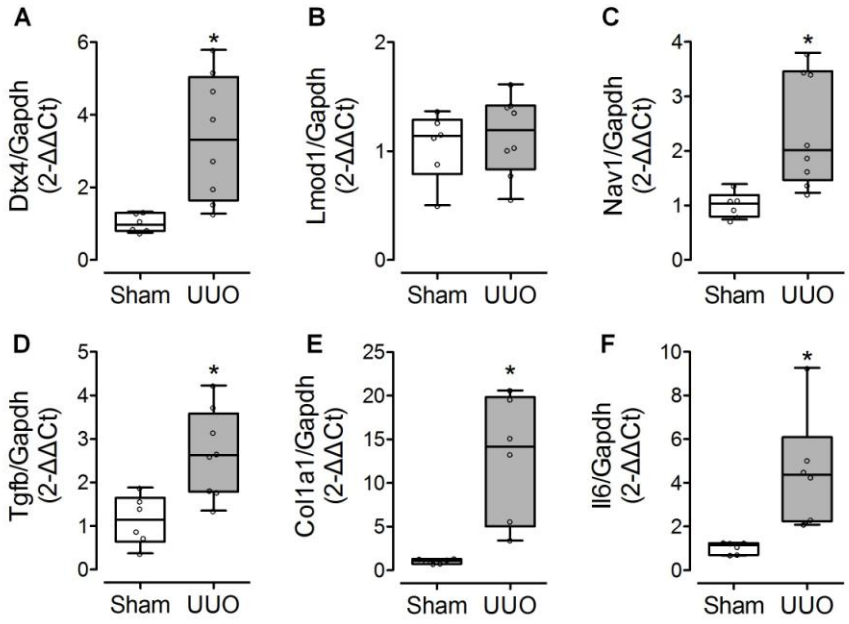

Figure 3. Selected mRNA predicted target expression in response to adult complete UUO *in vivo*. Renal expression of E3 ubiquitin-protein ligase DXT4 (Dtx4)(**A**), leiomodin-1 (Lmod1)(**B**), neuron navigator 1 (Nav1)(**C**), transforming growth factor beta (Tgfb)(**D**), collagen 1 (Col1a1)(**E**), and interleukin 6 (Il6)(**F**) was assessed by RT-PCR in adult mice after 7 days UUO.*$p<0.05$ versus Sham.

6.4. Discussion

Obstructive nephropathies, with UPJ obstruction as the prototypic obstructive nephropathy, are frequently encountered developmental anomalies in the pediatric population. Although renal fibrosis and inflammation are involved in the pathophysiology of severe UPJ obstruction, the molecular mechanisms underlying evolution of the lesions remain undetermined. In addition, most of this information has been obtained in animal models of disease. Recently discovered small non-coding RNAs called miRNAs are excreted and are stable in urine. Hence, urinary miRNAs could help to further decipher the pathophysiology of UPJ obstruction and generate the missing link between human and animal data. In this study we combined human urinary miRNA data and animal kidney tissue miRNA and mRNA data in a systems biology approach to obtain insight in the pathophysiology of the disease and improve upon the translatability of the results. The combined approach and independent validation pointed to the consistent dysregulation of specific miRNAs and to new potential targets in UPJ obstruction.

MiRNAs let-7a-5p, miR-125b-5p, miR-16-5p, miR-26a-5p and miR-29b-3p were consistently modified in mice and humans. Among these, miR-29b is a well-known player in renal pathologies and especially fibrosis. Indeed, miR-29b targets specific fibrotic molecules including collagens or β-smooth muscle actin, and its abundance is reduced in many fibrotic pathologies as its expression is inhibited by TGFβ **(Qin et al., 2011, Zhu et al., 2013)**. The protective role of miR-29b in fibrosis was further demonstrated *in vivo* since restoring miR-29b levels in a diabetic nephropathy animal model reversed accumulation of renal extracellular matrix **(Chen et al., 2014)**. On the other hand, to our knowledge, this is the first time that miR-16 and let-7a are associated to the development of kidney disease, as these miRNAs were mostly characterized to be involved in cancer **(Liu et al., 2012, Dong et al., 2010, Wang et al., 2013, Han et al., 2016, Chen et al., 2015a, Papadopoulos et al., 2015a)**. Downregulation of let-7a has also been observed in scleroderma, contributing to the excessive deposition of collagen and tissue fibrosis in the skin **(Makino et al., 2013)**. Few data is available for miR-125b and kidney disease. Circulating miR-125b was found downregulated in chronic kidney disease (CKD) in hypertensive patients suffering from cardiovascular disease **(Chen et al., 2015b)**. In another study upregulation of miR-125b protected against cisplatin-induced kidney injury via inhibition of Nuclear factor erythroid-2-related factor 2 **(Joo et al., 2013)**. MiR-26a has previously been reported to be over expressed in lung epithelia during development and to be involved in glomerular and tubular injury related to podocyte damage and maintenance of glomerular filtration rate **(Ho et al., 2008)**. A recent small-scale study (n=4 UPJ obstruction patients vs n=4 tumor-resection controls) identified in kidney tissue five miRNAs associated to UPJ obstruction. None of those overlapped

with the human urinary miRNAs in our study and one, hsa-miR-342-5p, was also found in kidney tissue to be associated to obstruction in the partial neonatal mouse model, however with an opposite regulation.

A total of 35 mRNA targets for these differentially expressed miRNAs were predicted with the use of Ingenuity Pathway Analysis. *In vitro* validation of the predicted miRNA-mRNA pairs and *in vivo* assessment of the observed mRNA changes led to the sound confirmation of the regulation of E3 ubiquitin-protein ligase DTX4 by let-7a. Although E3 ubiquitin-protein ligase DTX4 has not yet been found associated to obstructive nephropathy, using the Kidney & Urinary Pathway Knowledge Base (KUPKB, **(Klein et al., 2012)** we observed that it has been found to be induced in models of polycystic kidney disease **(Chen et al., 2008, Dweep et al., 2013)** and in a lupus nephritis model **(Teramoto et al., 2008)**. Furthermore, DTX4 is a member of Notch Signaling non-canonical pathway **(Viatour et al., 2011)**. It has been described that activation of Notch signaling can lead to fibrosis **(Sirin and Susztak, 2012, Lavoz et al., 2014, Djudjaj et al., 2012, Sanchez-Nino and Ortiz, 2012, Sweetwyne et al., 2014).** Hence one can hypothesize that downregulation of let-7a, activates DTX4 and the Notch signaling pathway, promoting the progression of fibrosis in UPJ obstruction.

NAV1 is a protein mostly found in neurons and is reported to play a critical role in microtubule development **(Martinez-Lopez et al., 2005)**, but has not yet been implicated in UPJ obstruction. Coinciding with DTX4, NAV1 expression is also induced in a model of polycystic kidney disease **(Chen et al., 2008)** and is induced *in vitro* by the major pro-fibrotic cytokine TGFβ **(Brennan et al., 2012)**, potentially linking NAV1 induction to fibrosis. It is notable, though, that the regulation of NAV1 by miR-29b-3p did not follow the classical regulation pattern (up regulation of a miRNA causes down regulation of a target or *vice versa*) in the partial UUO model. In contrast, in the *in vitro* experiment NAV1 followed the predicted regulation and confirming that it may be a direct target of miR-29b. Previous reports have documented that some miRNAs may not target mRNAs directly, but only block the protein translation, leaving the mRNA intact **(Nottrott et al., 2006, Petersen et al., 2006, Pillai et al., 2005, Pasquinelli, 2012)**. Furthermore, other reports point to the fact that miRNAs may induce the same direction of regulation of their mRNA targets depending on the timeframe and conditions **(Vasudevan, 2012, Vasudevan and Steitz, 2007, Vasudevan et al., 2007, Orom et al., 2008, Valinezhad Orang et al., 2014)**, providing an explanation of a possible indirect mechanism of miR-29b-NAV1 regulation. Nevertheless, further investigation is needed to determine if NAV1 is a direct target of miR-29b *in vivo* and if it is an interesting molecule in the context of UPJ obstruction.

Combination of the human urine miRNA data with the mouse tissue data increased the confidence in the human data and allowed selecting the most promising miRNAs in human disease. A downside of our approach is that this type of prioritization will only focus on part of the molecular mechanism of UPJ. If signals for specific mechanisms are absent in, for example, humans due to technical limitations or absence of shedding of miRNAs in urine but not in mouse tissue, this mechanism is not necessarily irrelevant in the pathophysiology of UPJ. Another shortcoming of our study is the fact that we used unadjusted p-values. Infants with UPJ obstruction included in the study displayed a wide range of hydronephrosis (grade 1-4, **Table 1**) and pelvic diameter (6-40 mm, **Table 1**) which generates considerable variability in the UPJ obstruction group. The mean fold change after comparison of healthy controls and UPJ obstruction patients was only 1.21 fold (± 0.27) in this data. This is probably due to the fact that during excretion/shedding of miRNAs in urine the *in-situ* miRNA changes are flattened out. Moreover, correction for multiple testing resulted in no significant miRNAs in humans. We therefore continued the prioritization with unadjusted pvalues but we compensated for this shortcoming by the independent validation of the selected targets.

In addition, we cannot be sure that the urinary miRNAs origin is from the kidney and not from other organ e.g. bladder, of the urinary system, but the combination of the human urine data with the mice tissue data (allowed by the ability of the miRNAs to be conserved between species) gives us high confidence. In our study, we did not observe enriched canonical pathways when studying the miRNAs of human or mouse UPJ obstruction separately even if around hundreds of miRNAs were found significantly different. Hence we have used both statistical selection, combination of different omics data (miRNA and mRNA data) and pathway enrichment analysis to identify miRNAs and their targets most likely involved in the etiology of UPJ obstruction.

6.5. Conclusion

Collectively this study is the first to correlate a mouse model of neonatal partial UUO with human UPJ obstruction in a comprehensive systems biology analysis. Our data revealed let-7a and miR-29b as molecules potentially involved in the development of fibrosis in UPJ obstruction via the control of DTX4 in both man and mice that would not be identified otherwise.

We believe that our approach of combining omics data is generally applicable. Many omics studies generate long lists of differentially expressed molecules that are difficult to prioritize and does not necessarily inform on the actual impact of these changes in disease. To further improve on the validity and clinical translatability of the data, and because urine is a rich source of biomarkers that can be collected easily and non-invasively, combination of human urine

and animal tissue data (e.g. miRNA, metabolites, proteins etc) could be of great help to better understand the molecular mechanisms involved in the development of many complex diseases.

6.6. Materials and Methods

Human samples

The studies were performed in accordance with the ethical principles in the Declaration of Helsinki and Good Clinical Practice and was approved by the CPP SOOM II (number DC-2008-452). Written informed consent was obtained from all participants (parents). The UPJO group was composed of patients with grade 1 to 4 hydronephrosis and a renal pelvic diameter between 6 and 40 mm (**Table 1**). Urine was collected from boys (<1 y). Urine from patients was collected in the morning during 30 min using a sterile pediatric urine collection pouch (B. Braun, Boulogne, France) during hospital consultation. Samples from healthy controls were both collected in a hospital setting (from newborns with heart murmur) and at home. Immediately after collection, all urines were frozen at -20°C and stored at -80°C.

Mouse models

All mouse experiments were conducted in accordance with the NIH guide for the care and use of laboratory animals and were approved by the University of Virginia Animal Care and Use Committee (for the neonatal partial obstruction model) and the animal care and use committee UMS US006/INSERM, Toulouse, France (protocol number CEEA-122 2014-06 / 02605.01) for the complete adult obstruction model.

Both the partial neonatal and adult complete unilateral ureteral obstruction (UUO) mouse models have been previously described **(Thornhill et al., 2007, Klein et al., 2009)**. Neonatal mice and adult mice were sacrificed after 5-7 and 7 days of obstruction, respectively.

Microarray analysis

Microarray analysis was carried out as described previously **(Bachvarov et al., 2006, Faddaoui et al., 2016).** Briefly, total RNA was extracted from kidney tissues of 9 neonatal mice with partial UUO (hydronephrosis grades 2 and 3) and 9 control sham operated mice. Fluorescently labeled cRNA targets were generated using the Fluorescent Linear Amplification Kit (Agilent) and 10.0 mM Cyanine 3- or 5-labeled CTP (PerkinElmer, Boston, MA), and following user's manual. Cyanine labeled cRNA from UUO kidneys was mixed with the same amount of reverse-color cyanine-labeled cRNA from the corresponding control kidney samples and hybridized on the Agilent 44K Mouse Whole Genome Oligonucleotide Microarray. Array hybridization, washing and scanning were performed as previously described **(Bachvarov et al., 2006, Faddaoui et al., 2016)**.

MicroRNA expression profiling was performed using the Mouse miRNA Microarray Release 15.0 (8×15K, G4471A-029152, Agilent Technologies), and the Human miRNA V3 Microarray Release 12.0 (8×15K, G44710C-021827, Agilent Technologies). Briefly, 100 ng of total RNA was labeled and hybridized using the commercial miRNA Microarray System with miRNA Complete and Hybridization Kit (Agilent Technologies) following manufacturer's instructions. Array hybridization, washing, scanning, data extraction, and analyses were performed as described previously.

Cell models and antagomir treatment

Human HK-2 cells were grown in a DMEM/F-12 Nut Mix medium supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO, Grand Island, NY, USA), 10 µg/mL of EGF, 5 µg/mL of insulin, 4 pg/mL Triiodothyronine (T3), 36ng/mL of hydrocortisone. After 24 hours of FCS starvation, HK-2 cells were transfected with 5 nmol IDT® miRNA inhibitor targeting 5 miRNAs: hsa-let-7a-5p (ref. no 67488991), hsa-miR-125b-5p (ref. no 67488990) hsa-miR-16-5p (ref no. 67488992), hsa-miR-26a-5p (ref. no. 67488993) and hsa-miR-29b-3p (ref. no. 67488994), or with scrambled siRNA (Integrated DNA Technologies, Leuven, Belgium), using the DharmaFECT Duo transfection reagent (Dharmacon, Lafayette, CO, USA).

Gene expression analysis

Total RNA was extracted from human kidney cells (HK2 cells) and complete UUO kidney samples using the Illumina's Epicentre MasterPure Kit (Madison, WI, USA). Reverse transcription was performed for the miRNAs with MiRCURY LNA Universal RT Kit of Exiqon (Vedbaek, Denmark) and for the mRNA with High Capacity cDNA Reverse Transcription Kit of Thermo Scientific (Waltham, MA, USA) on a FlexCycler2 (Analytik Jena AG, Jena, Germany). Quantitative PCR amplification was performed on a StepOnePlus Real-Time PCR System (Thermo Scientific Waltham, MA, USA). Sybr Green technology was used for miRNAs according to Exiqon's kit, while for the mRNA PCR the MESA BLUE qPCR MasterMix Plus kit from Eurogentec (Liege, Belgium). The primers used for the PCR are listed in **Additional File 9**.

Bioinformatic analysis

Network and pathway analysis of the microarray data were performed using Ingenuity Pathway Analysis (IPA) software version 18488943 (IPA®, QIAGEN Redwood City, see http://www.Ingenuity.com).

Statistics

For the microarrays statistical analysis the freely available software Gene ARMADA was used **(Chatziioannou et al., 2009)**. A background correction was made for all arrays by loess correction and normalization was made by linear lowess followed by quantile normalization. The statistically different genes were the result of a t-test analysis (p-value < 0.05). The results of the qPCR for the cell cultures and the partial and complete UUO experiments were expressed in fold change units based on $2^{-\Delta\Delta Ct}$ method. The graphs and the statistical analysis (Mann - Whitney test between the groups with p-value < 0.05 as significant) were performed with GraphPad Prism v5.0.

6.7. DECLARATIONS

Ethics approval

The studies were performed in accordance with the ethical principles in the Declaration of Helsinki and Good Clinical Practice and was approved by the CPP SOOM II (number DC-2008-452). Written informed consent was obtained from all participants (parents).

All mouse experiments were conducted in accordance with the NIH guide for the care and use of laboratory animals and were approved by the University of Virginia Animal Care and Use Committee (for the neonatal partial obstruction model) and the animal care and use committee UMS US006/INSERM, Toulouse, France (protocol number CEEA-122 2014-06 / 02605.01) for the complete adult obstruction model.

Consent for publication

Not applicable.

Availability of data material

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TP, JK and JPS conceived the study, analyzed the data and wrote the manuscript; AC, CC and JB performed *in-vitro* experiments; MB, DB, JLB and BBM performed microarray experiments; DC, BAT and RLC performed animal experiments; PM and EN performed statistical analysis; SD, FCA and BB collected clinical data and banked human material. All authors reviewed the manuscript.

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Not applicable.

6.8 Additional Data

Additional Table 1: Differentially expressed miRNAs in human UPJ.

Symbol	Exp Fold Change	Exp p-value	Symbol	Exp Fold Change	Exp p-value
Down-regulated					
mir-638	-3,467	2,29E-02	mir-639	-1,118	7,93E-03
miR-188-5p	-3,161	4,21E-02	miR-142-3p	-1,116	2,65E-03
miR-574-5p	-2,9	6,73E-03	miR-30a-3p	-1,116	1,00E-02
miR-574-3p	-2,439	2,54E-03	miR-644a	-1,115	9,16E-03
miR-483-5p	-2,203	4,66E-02	miR-432-3p	-1,113	1,62E-02
mir-21	-1,948	1,20E-02	miR-18a-3p	-1,112	4,23E-04
mir-572	-1,893	1,73E-02	mir-449	-1,112	4,05E-02
mir-601	-1,842	8,93E-03	miR-550a-3p	-1,112	3,86E-02
miR-671-5p	-1,698	3,01E-02	miR-92a-2-5p	-1,112	6,25E-03
mir-10	-1,645	1,08E-02	miR-941	-1,11	2,72E-02
mir-765	-1,586	1,74E-03	mir-647	-1,107	2,48E-02
let-7a-5p	-1,559	3,17E-03	miR-7a-5p	-1,107	1,09E-02
MIR659	-1,545	2,09E-02	miR-31-3p	-1,104	8,13E-03
miR-135a-1-3p	-1,443	2,78E-02	miR-194-3p	-1,101	1,36E-02
miR-26a-5p	-1,376	3,03E-02	miR-34a-5p	-1,101	8,55E-03
mir-204	-1,348	1,68E-02	miR-140-5p	-1,1	5,27E-03
miR-483-3p	-1,344	1,30E-02	miR-369-3p	-1,1	3,71E-03
miR-32-3p	-1,343	7,61E-03	mir-612	-1,098	2,17E-02
mir-708	-1,336	4,21E-03	miR-548f-3p	-1,096	2,50E-02
mir-596	-1,329	9,72E-05	miR-148b-5p	-1,094	4,30E-03
mir-8	-1,328	3,15E-02	miR-27a-5p	-1,089	7,76E-03
mir-22	-1,31	2,18E-02	mir-302	-1,089	3,12E-02
miR-1228-5p	-1,306	3,42E-02	miR-590-3p	-1,089	4,73E-02
miR-16-5p	-1,293	2,27E-03	mir-652	-1,089	6,27E-03
mir-34	-1,271	3,42E-03	miR-767	-1,089	1,47E-02
miR-1224-3p	-1,264	2,50E-02	miR-34c-3p	-1,088	2,40E-02
mir-26	-1,263	1,99E-03	miR-17-3p	-1,087	2,75E-02
mir-23	-1,257	1,39E-02	miR-485-5p	-1,087	4,96E-02
miR-518c-5p	-1,256	4,20E-06	mir-373	-1,081	3,15E-02
let-7	-1,255	3,92E-04	miR-505-5p	-1,081	2,59E-02
miR-10b-3p	-1,246	5,22E-03	mir-609	-1,08	3,61E-02
miR-509-3p	-1,244	8,27E-03	mir-580	-1,078	2,40E-02
mir-27	-1,241	5,70E-03	mir-675	-1,078	4,03E-02
mir-322	-1,233	3,15E-03	miR-1273h-5p	-1,074	4,41E-02
mir-17	-1,218	2,72E-05	miR-885-3p	-1,064	4,91E-02

Symbol	Exp Fold Change	Exp p-value	Symbol	Exp Fold Change	Exp p-value
mir-30	-1,214	2,43E-02	miR-337-5p	-1,06	2,89E-02
mir-328	-1,212	1,12E-02	mir-122	-1,059	4,26E-02
mir-29	-1,205	1,10E-02	mir-619	-1,118	8,27E-03
mir-146	-1,201	8,54E-04	miR-299a-3p	-1,119	5,09E-03
miR-195-3p	-1,188	2,99E-02	miR-29b-3p	-1,153	2,93E-02
miR-19b-3p	-1,188	4,13E-02	miR-27b-5p	-1,151	3,18E-03
mir-130	-1,186	1,24E-02	miR-194-5p	-1,145	1,63E-02
miR-203a-3p	-1,186	1,17E-02	miR-135a-5p	-1,14	2,85E-02
miR-92a-3p	-1,185	3,93E-03	miR-10a-3p	-1,139	1,10E-02
miR-125b-5p	-1,18	3,03E-02	mir-210	-1,138	2,94E-02
miR-628-3p	-1,18	8,60E-05	mir-325	-1,138	1,37E-02
mir-297	-1,177	3,75E-03	mir-561	-1,137	2,48E-02
mir-187	-1,17	1,24E-03	miR-526a	-1,136	7,87E-03
miR-149-3p	-1,167	3,53E-02	miR-103-3p	-1,135	6,67E-03
mir-103	-1,164	3,81E-03	mir-581	-1,134	1,38E-02
mir-148	-1,162	4,35E-03	mir-221	-1,133	1,12E-03
miR-151-5p	-1,162	9,09E-03	mir-383	-1,132	5,93E-03
mir-432	-1,16	8,83E-03	miR-101-3p	-1,13	3,98E-03
mir-15	-1,159	1,85E-02	mir-31	-1,13	3,83E-03
mir-192	-1,159	6,06E-03	miR-138-5p	-1,129	4,33E-03
mir-571	-1,155	1,03E-02	mir-19	-1,128	1,14E-02
miR-376a-5p	-1,122	7,03E-03	miR-532-5p	-1,128	2,49E-03
miR-9-5p	-1,121	3,47E-03	miR-219a-2-3p	-1,127	7,56E-03
mir-25	-1,119	7,90E-03	mir-626	-1,127	1,23E-03
Up-regulated					
miR-18b-3p	1,051	4,35E-02	miR-187-5p	1,274	2,71E-02
mir-183	1,056	4,06E-02	miR-425-3p	1,274	1,79E-02
miR-146a-3p	1,058	2,64E-02	mir-627	1,291	2,91E-03
miR-455-3p	1,066	4,69E-02	mir-634	1,311	4,03E-02
mir-368	1,07	2,64E-02	miR-191-3p	1,345	7,21E-03
miR-15b-3p	1,077	1,90E-02	miR-541-3p	1,415	2,80E-02
miR-106b-3p	1,078	4,41E-02	miR-362-5p	1,478	2,88E-02
miR-1231-3p	1,079	7,39E-03	mir-181	1,613	4,97E-02
mir-217	1,081	4,11E-02	mir-631	1,945	4,91E-02
miR-628	1,082	4,80E-02	mir-874	3,275	2,06E-02
miR-624-5p	1,084	4,49E-02	mir-643	1,205	7,48E-03

Symbol	Exp Fold Change	Exp p-value	Symbol	Exp Fold Change	Exp p-value
miR-377-5p	1,085	3,67E-02	miR-193a-3p	1,211	1,42E-02
mir-668	1,088	1,27E-02	mir-154	1,216	7,24E-03
miR-450b-5p	1,089	1,41E-02	miR-129-1-3p	1,241	1,56E-03
mir-191	1,092	1,37E-02	mir-887	1,249	4,31E-03
miR-514a-3p	1,094	4,84E-03	miR-376a-3p	1,259	1,11E-03
miR-100-3p	1,095	4,26E-02	miR-365-3p	1,27	2,22E-02
miR-138-2-3p	1,095	4,89E-02	miR-7-1-3p	1,142	4,19E-02
miR-501-3p	1,095	2,81E-02	miR-124-5p	1,144	4,57E-03
miR-24-1-5p	1,096	8,30E-03	miR-133a-3p	1,144	2,32E-03
mir-425	1,096	9,06E-03	mir-555	1,145	3,71E-02
miR-144-5p	1,097	1,57E-03	mir-607	1,146	7,35E-03
miR-127-5p	1,098	2,05E-02	mir-661	1,146	4,51E-02
miR-593-5p	1,098	4,16E-02	mir-877	1,146	4,42E-02
mir-379	1,101	3,60E-02	miR-532-3p	1,147	1,23E-02
miR-875-5p	1,101	4,08E-02	miR-26b-3p	1,148	1,13E-02
miR-182-3p	1,102	6,80E-03	mir-552	1,149	8,86E-03
miR-488-5p	1,103	2,83E-03	miR-218-1-3p	1,15	1,15E-03
miR-582-5p	1,103	1,40E-02	miR-223-5p	1,15	1,84E-02
mir-133	1,105	7,67E-03	mir-566	1,152	1,70E-02
miR-486-5p	1,106	8,87E-03	mir-551	1,154	3,55E-03
miR-219a-5p	1,11	3,40E-02	mir-597	1,154	6,21E-03
miR-34a-3p	1,113	8,73E-03	MIR1229	1,157	3,62E-02
mir-548	1,113	1,26E-02	miR-218-2-3p	1,157	1,60E-02
mir-635	1,113	1,44E-03	miR-192-3p	1,161	1,96E-02
miR-202-5p	1,114	4,57E-02	miR-520a-5p	1,163	5,47E-03
mir-412	1,116	3,68E-02	mir-891	1,163	4,24E-03
mir-32	1,118	5,03E-03	mir-943	1,164	2,10E-03
miR-515-5p	1,118	7,06E-03	mir-588	1,17	3,40E-03
mir-489	1,121	7,94E-03	miR-4712-5p	1,175	2,47E-02
mir-506	1,121	1,06E-02	mir-216	1,178	4,94E-02
miR-153-3p	1,124	2,51E-02	mir-150	1,179	4,25E-02
miR-509-5p	1,124	7,95E-03	miR-518a-3p	1,182	4,89E-02
miR-642a-5p	1,127	3,73E-02	miR-329-3p	1,19	7,54E-03
miR-1233-3p	1,128	2,06E-02	mir-515	1,191	2,34E-02
mir-149	1,128	4,20E-02	miR-409-3p	1,193	3,57E-04
miR-548c-3p	1,128	3,19E-03	miR-615-5p	1,194	1,08E-05
miR-625-3p	1,128	4,09E-02	mir-126	1,197	3,38E-02

Symbol	Exp Fold Change	Exp p-value	Symbol	Exp Fold Change	Exp p-value
mir-567	1,129	4,42E-02	miR-361-5p	1,198	2,57E-02
miR-542-3p	1,13	3,27E-02	miR-767-3p	1,2	4,52E-03
mir-935	1,13	2,39E-03	mir-920	1,204	1,76E-03
miR-138-1-3p	1,132	2,34E-02	mir-1231	1,142	2,36E-02
miR-145-3p	1,134	4,68E-03	mir-653	1,142	3,32E-02
miR-340-3p	1,134	3,86E-02			
miR-221-5p	1,137	3,51E-02			
miR-671-3p	1,139	1,45E-02			

Additional Table 2: Differentially expressed miRNAs in mouse partial UUO.

Symbol	Exp Fold Change	Exp p-value	Symbol	Exp Fold Change	Exp p-value
Down-regulated					
miR-690	-5,186	3,80E-03	miR-148a-3p	-1,702	2,11E-02
miR-466b-5p	-4,151	7,82E-03	miR-140-5p	-1,69	4,05E-03
miR-1827	-4,035	1,30E-02	miR-378a-3p	-1,687	5,88E-03
let-7a-5p	-3,558	3,93E-03	miR-342-3p	-1,673	3,01E-03
miR-143-3p	-3,332	3,72E-04	miR-146a-5p	-1,671	3,30E-03
miR-125b-5p	-3,219	3,15E-03	miR-126a-5p	-1,649	1,11E-02
miR-26a-5p	-3,175	1,13E-03	miR-708-5p	-1,609	4,41E-03
miR-199a-5p	-3,087	2,18E-03	miR-139-5p	-1,431	2,50E-02
miR-466f-3p	-3,079	2,13E-02	miR-101b-3p	-1,39	1,68E-02
miR-10a-5p	-3,052	4,39E-03	miR-296-5p	-1,305	7,39E-04
miR-181a-5p	-3,028	1,54E-02	miR-344a-5p	-1,286	1,37E-02
miR-16-5p	-2,913	6,97E-04	let-7d-3p	-1,28	2,83E-05
miR-3941	-2,901	8,56E-03	miR-330-5p	-1,277	4,20E-02
miR-466g	-2,815	1,42E-02	miR-615-3p	-1,274	3,37E-05
miR-467a-3p	-2,737	1,35E-02	miR-328-3p	-1,228	3,35E-04
miR-196a-5p	-2,729	1,25E-03	miR-186-5p	-1,222	1,80E-03
miR-23a-3p	-2,674	8,97E-05	miR-193a-3p	-1,221	2,70E-03
miR-214-3p	-2,655	1,04E-02	miR-203a-3p	-1,198	1,21E-02
miR-17-5p	-2,508	4,05E-03	miR-149-5p	-1,186	1,11E-02
miR-19b-3p	-2,448	1,89E-03	miR-425-5p	-1,183	1,48E-02
miR-130a-3p	-2,363	4,00E-03	miR-10a-3p	-1,174	4,67E-03
miR-103-3p	-2,179	6,54E-03	miR-181a-1-3p	-1,172	6,81E-03
miR-141-3p	-2,167	3,48E-03	miR-744-5p	-1,165	3,13E-03
miR-27a-3p	-2,155	2,10E-03	miR-669a-5p	-1,15	1,41E-02
miR-30c-5p	-2,151	1,35E-02	miR-320b	-1,149	7,42E-03
miR-218-5p	-2,146	3,27E-03	miR-132-3p	-1,108	2,30E-02
miR-151-5p	-2,144	8,04E-03	miR-338-5p	-1,069	1,01E-05
miR-652-3p	-2,111	1,02E-02	miR-3909	-1,066	6,45E-04
miR-92a-3p	-2,076	8,14E-03	miR-487b-3p	-1,041	3,56E-14
miR-145-5p	-2,056	1,73E-03	miR-106b-3p	-1,04	8,56E-05
miR-1895	-2,046	4,82E-03	miR-467b-5p	-1,028	2,66E-04
miR-24-3p	-1,96	6,50E-03	miR-151-3p	-1,005	6,31E-04
miR-100-5p	-1,952	1,32E-02	miR-331-3p	-1,9	1,35E-02
miR-455-3p	-1,933	1,34E-02	miR-126a-3p	-1,846	1,63E-02
miR-221-3p	-1,931	3,96E-03	miR-200b-3p	-1,841	1,12E-02

miR-142-3p	-1,93	3,03E-03	miR-345-5p	-1,809	2,80E-03
miR-365-3p	-1,928	1,45E-02			
Up-regulated					
miR-9-3p	1,006	1,32E-03	miR-452-5p	1,131	2,59E-05
miR-20a-3p	1,035	3,55E-04	miR-29b-3p	10,073	3,10E-08
miR-24-1-5p	1,05	8,59E-04	miR-224-5p	1,08	1,73E-06

Additional Table 3: Differentially expressed mRNAs in mouse partial UUO.

Gene Symbol	Description	Exp Fold Change	Exp p-value
Down-regulated			
Aqp4	aquaporin 4	-3,45	0,00111702
Egf	epidermal growth factor	-3,23	0,00002300
Cfd	complement factor D (adipsin)	-3,03	0,00171642
Cidea	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	-2,78	0,00061255
Cox8b	cytochrome c oxidase, subunit VIIIb	-2,63	0,00358692
Ptger3	prostaglandin E receptor 3 (subtype EP3)	-2,63	0,00003000
Aadat	aminoadipate aminotransferase	-2,50	0,00560964
F13b	coagulation factor XIII, beta subunit	-2,44	0,00000551
Abca13	ATP-binding cassette, sub-family A (ABC1), member 13	-2,38	0,00214086
Slc14a2	solute carrier family 14 (urea transporter), member 2	-2,38	0,00100433
C11orf54	chromosome 11 open reading frame 54	-2,27	0,00080400
Efhd1	EF hand domain containing 1	-2,22	0,00228723
HLA-A	major histocompatibility complex, class I, A	-2,17	0,00000121
Mthfd1	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase	-2,17	0,00005360
Pah	phenylalanine hydroxylase	-2,17	0,00001400
Glod5	glyoxalase domain containing 5	-2,17	0,00000855
Ppp1r1a	protein phosphatase 1, regulatory (inhibitor) subunit 1A	-2,13	0,00629651
Slc16a7	solute carrier family 16 (monocarboxylic acid transporters), member 7	-2,13	0,00544090
Acsl1	acyl-CoA synthetase long-chain family member 1	-2,13	0,00174555
Slc6a19	solute carrier family 6 (neurotransmitter transporter), member 19	-2,13	0,00193457
Slc34a3	solute carrier family 34 (sodium phosphate), member 3	-2,13	0,00012044
Cldn8	claudin 8	-2,08	0,00377068
Calb1	calbindin 1	-2,08	0,00262563
Klf15	Kruppel-like factor 15	-2,08	0,00242003
Kcnj10	potassium inwardly-rectifying channel, subfamily J, member 10	-2,08	0,00116043
Slco1a6	solute carrier organic anion transporter family, member 1a6	-2,08	0,00127739
Sult1d1	sulfotransferase family 1D, member 1	-2,08	0,00092928
Dnase1	deoxyribonuclease I	-2,08	0,00056505
Apoo	apolipoprotein O	-2,08	0,00003190
Fut9	fucosyltransferase 9	-2,08	0,00000160
NAT8B	N-acetyltransferase 8B (GCN5-related, putative, gene/pseudogene)	-2,08	0,00134000
KLK3	kallikrein-related peptidase 3	-2,08	0,00077500
DEFB119	defensin, beta 119	-2,08	0,00001250
Atp6v0a4	ATPase, H+ transporting, lysosomal V0 subunit A4	-2,04	0,00876550
Slc5a8	solute carrier family 5 (iodide transporter), member 8	-2,04	0,00528178
Gm13111	predicted gene 13111	-2,04	0,00217481

Slc16a4	solute carrier family 16 (monocarboxylic acid transporters), member 4	-2,04	0,00051605
Slc26a4	solute carrier family 26, member 4	-2,04	0,00016218
Slc5a3	solute carrier family 5 (inositol transporters), member 3	-2,04	0,00018154
Slc9a3	solute carrier family 9 (sodium/hydrogen exchanger), member 3	-2,04	0,00005490
S100g	S100 calcium binding protein G	-2,00	0,00586946
Slco1a1	solute carrier organic anion transporter family, member 1a1	-2,00	0,00234929
Adhfe1	alcohol dehydrogenase, iron containing, 1	-2,00	0,00155576
Acy1	aminoacylase 1	-2,00	0,00087079
ACSM2A	acyl-CoA synthetase medium-chain family member 2A	-2,00	0,00015300
MPC1	mitochondrial pyruvate carrier 1	-2,00	0,00010700
KIAA1919	KIAA1919	-1,96	0,00320000
Clec2e/Clec2h	C-type lectin domain family 2, member h	-1,96	0,00168000
Gk	glycerol kinase	-1,96	0,00175000
Slc22a7	solute carrier family 22 (organic anion transporter), member 7	-1,96	0,00389173
Ass1	argininosuccinate synthetase 1	-1,96	0,00228847
Fam151a	family with sequence simliarity 151, member A	-1,96	0,00215228
Igsf11	immunoglobulin superfamily, member 11	-1,96	0,00101758
Pla1a	phospholipase A1 member A	-1,96	0,00078673
Mccc1	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	-1,96	0,00034670
Mpv17l	Mpv17 transgene, kidney disease mutant-like	-1,96	0,00027342
Slc2a5	solute carrier family 2 (facilitated glucose transporter), member 5	-1,96	0,00005880
4931408D14Rik	RIKEN cDNA 4931408D14 gene	-1,92	0,00352076
Acox2	acyl-Coenzyme A oxidase 2, branched chain	-1,92	0,00317214
Hao2	hydroxyacid oxidase 2	-1,92	0,00128253
Bdh1	3-hydroxybutyrate dehydrogenase, type 1	-1,92	0,00013154
Hgd	homogentisate 1, 2-dioxygenase	-1,92	0,00080256
Smpx	small muscle protein, X-linked	-1,92	0,00055296
Cav2	caveolin 2	-1,92	0,00019344
Lrrc19	leucine rich repeat containing 19	-1,92	0,00012931
CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	-1,89	0,00427000
TMEM52B	transmembrane protein 52B	-1,89	0,00011400
Col4a3	collagen, type IV, alpha 3	-1,89	0,00739556
Slc2a9	solute carrier family 2 (facilitated glucose transporter), member 9	-1,89	0,00446636
Slc37a4	solute carrier family 37 (glucose-6-phosphate transporter), member 4	-1,89	0,00387738
Slc22a8	solute carrier family 22 (organic anion transporter), member 8	-1,89	0,00368034
Acot4	acyl-CoA thioesterase 4	-1,89	0,00314130
Cth	cystathionase (cystathionine gamma-lyase)	-1,89	0,00266431
Lactb2	lactamase, beta 2	-1,89	0,00256119
Mep1a	meprin 1 alpha	-1,89	0,00243824

Sult1b1	sulfotransferase family 1B, member 1	-1,89	0,00268569
Lypla1	lysophospholipase 1	-1,89	0,00136623
Dmgdh	dimethylglycine dehydrogenase precursor	-1,89	0,00097338
Aldh6a1	aldehyde dehydrogenase family 6, subfamily A1	-1,89	0,00049530
Slc12a3	solute carrier family 12, member 3	-1,89	0,00045364
Tcn2	transcobalamin 2	-1,89	0,00035310
Kcnj15	potassium inwardly-rectifying channel, subfamily J, member 15	-1,89	0,00016787
Slc16a12	solute carrier family 16 (monocarboxylic acid transporters), member 12	-1,89	0,00018462
Slc5a12	solute carrier family 5 (sodium/glucose cotransporter), member 12	-1,89	0,00023751
FAM195A	family with sequence similarity 195, member A	-1,85	0,00722000
CFAP52	cilia and flagella associated protein 52	-1,85	0,00037700
CYP2A6 (includes others)	cytochrome P450, family 2, subfamily A, polypeptide 6	-1,85	0,00031100
Igfbp2	insulin-like growth factor binding protein 2	-1,85	0,00391132
Retsat	retinol saturase (all trans retinol 13,14 reductase)	-1,85	0,00527904
Exoc6	exocyst complex component 6	-1,85	0,00392703
Cox7a1	cytochrome c oxidase, subunit VIIa 1	-1,85	0,00020395
Dio1	deiodinase, iodothyronine, type I	-1,85	0,00101572
Npnt	nephronectin	-1,85	0,00068332
Acaa2	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	-1,85	0,00001060
Folh1	folate hydrolase	-1,82	0,00633490
Cldn10	claudin 10	-1,82	0,00409089
Bckdhb	branched chain ketoacid dehydrogenase E1, beta polypeptide	-1,82	0,00341395
Slc4a1	solute carrier family 4 (anion exchanger), member 1	-1,82	0,00293392
Slc5a2	solute carrier family 5 (sodium/glucose cotransporter), member 2	-1,82	0,00197650
Tmed6	transmembrane emp24 protein transport domain containing 6	-1,82	0,00232168
Agxt2	alanine-glyoxylate aminotransferase 2	-1,82	0,00113143
Ttc36	tetratricopeptide repeat domain 36	-1,82	0,00011848
Fam13a	family with sequence similarity 13, member A	-1,82	0,00054200
PC	pyruvate carboxylase	-1,82	0,00020700
LRP2	low density lipoprotein receptor-related protein 2	-1,82	0,00004590
CA14	carbonic anhydrase XIV	-1,79	0,00290000
ACOT1	acyl-CoA thioesterase 1	-1,79	0,00218000
Hnf4aos	hepatic nuclear factor 4 alpha, opposite strand	-1,79	0,00007630
Nepn	nephrocan	-1,79	0,00438063
Slc27a2	solute carrier family 27 (fatty acid transporter), member 2	-1,79	0,00487881
Wscd2	WSC domain containing 2	-1,79	0,00449274
Atp6v1c2	ATPase, H+ transporting, lysosomal V1 subunit C2	-1,79	0,00290917
Dynll2	dynein light chain LC8-type 2	-1,79	0,00097154
Hif1a	hypoxia inducible factor 1, alpha subunit	-1,79	0,00115289

Proz	protein Z, vitamin K-dependent plasma glycoprotein	-1,79	0,00124629
Ednrb	endothelin receptor type B	-1,79	0,00063445
Tfrc	transferrin receptor	-1,79	0,00074885
Tpk1	thiamine pyrophosphokinase	-1,79	0,00027421
Chpt1	choline phosphotransferase 1	-1,79	0,00025737
Spp2	secreted phosphoprotein 2	-1,79	0,00007470
Acss1	acyl-CoA synthetase short-chain family member 1	-1,79	0,00002130
Scel	sciellin	-1,75	0,00650457
Mro	maestro	-1,75	0,00319368
Susd2	sushi domain containing 2	-1,75	0,00417487
Slc25a13	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 13	-1,75	0,00320486
Slc5a11	solute carrier family 5 (sodium/glucose cotransporter), member 11	-1,75	0,00114186
Lrrc66	leucine rich repeat containing 66	-1,75	0,00089267
Slc39a8	solute carrier family 39 (metal ion transporter), member 8	-1,75	0,00101403
Fn3k	fructosamine 3 kinase	-1,75	0,00007400
Angpt1	angiopoietin 1	-1,75	0,00051827
Kcnt1	potassium channel, subfamily T, member 1	-1,75	0,00032549
Cgref1	cell growth regulator with EF hand domain 1	-1,75	0,00008990
Cycs	cytochrome c, somatic	-1,75	0,00010120
Grhpr	glyoxylate reductase/hydroxypyruvate reductase	-1,75	0,00015128
Pdzk1	PDZ domain containing 1	-1,75	0,00013139
Gas2	growth arrest specific 2	-1,75	0,00002360
Gcsh	glycine cleavage system protein H (aminomethyl carrier)	-1,75	0,00001720
Atp6v1e1	ATPase, H+ transporting, lysosomal V1 subunit E1	-1,75	0,00000466
TMEM242	transmembrane protein 242	-1,75	0,00374000
MTFP1	mitochondrial fission process 1	-1,75	0,00081700
Col4a5	collagen, type IV, alpha 5	-1,72	0,00726260
Dnajc12	DnaJ (Hsp40) homolog, subfamily C, member 12	-1,72	0,00339041
Mogat2	monoacylglycerol O-acyltransferase 2	-1,72	0,00353556
Hspa4	heat shock protein 4	-1,72	0,00146002
Npl	N-acetylneuraminate pyruvate lyase	-1,72	0,00255782
Miox	myo-inositol oxygenase	-1,72	0,00029527
Tpd52l1	tumor protein D52-like 1	-1,72	0,00073787
Serpina6	serine (or cysteine) peptidase inhibitor, clade A, member 6	-1,72	0,00053010
Slc2a2	solute carrier family 2 (facilitated glucose transporter), member 2	-1,72	0,00058582
Hnf4a	hepatic nuclear factor 4, alpha	-1,72	0,00035201
Arhgap24	Rho GTPase activating protein 24	-1,72	0,00020558
Sdhb	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	-1,72	0,00018012
Mdh1	malate dehydrogenase 1, NAD (soluble)	-1,72	0,00010605

Aga	aspartylglucosaminidase	-1,72	0,00000847
SLC22A25	solute carrier family 22, member 25	-1,70	0,00158000
MT-ATP6	ATP synthase F0 subunit 6	-1,70	0,00035000
Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	-1,69	0,00432072
Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	-1,69	0,00317024
Slc6a12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	-1,69	0,00311852
Crim1	cysteine rich transmembrane BMP regulator 1 (chordin like)	-1,69	0,00033293
Dlst	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	-1,69	0,00127817
Sgk1	serum/glucocorticoid regulated kinase 1	-1,69	0,00113983
Grpel1	GrpE-like 1, mitochondrial	-1,69	0,00094813
Rmnd1	required for meiotic nuclear division 1 homolog (S, cerevisiae)	-1,69	0,00088226
Eaf2	ELL associated factor 2	-1,69	0,00070345
Pou3f3	POU domain, class 3, transcription factor 3	-1,69	0,00051809
Fxyd4	FXYD domain-containing ion transport regulator 4	-1,69	0,00019303
Aifm1	apoptosis-inducing factor, mitochondrion-associated 1	-1,69	0,00003160
Ankib1	ankyrin repeat and IBR domain containing 1	-1,69	0,00002840
Chchd10	coiled-coil-helix-coiled-coil-helix domain containing 10	-1,69	0,00004000
Olfr784/Olfr801	olfactory receptor 784	-1,67	0,00680000
MCUR1	mitochondrial calcium uniporter regulator 1	-1,67	0,00075300
Bhmt2	betaine-homocysteine methyltransferase 2	-1,67	0,00465821
Tal2	T-cell acute lymphocytic leukemia 2	-1,67	0,00606718
Afp	alpha fetoprotein	-1,67	0,00532478
Cd36	CD36 antigen	-1,67	0,00531177
Itga8	integrin alpha 8	-1,67	0,00510390
Fabp4	fatty acid binding protein 4, adipocyte	-1,67	0,00469567
Itgb6	integrin beta 6	-1,67	0,00279674
Abhd3	abhydrolase domain containing 3	-1,67	0,00427509
Dusp9	dual specificity phosphatase 9	-1,67	0,00211848
Rab3ip	RAB3A interacting protein	-1,67	0,00349749
Dld	dihydrolipoamide dehydrogenase	-1,67	0,00327237
Sectm1b	secreted and transmembrane 1B	-1,67	0,00325139
Habp2	hyaluronic acid binding protein 2	-1,67	0,00260458
Mal	myelin and lymphocyte protein, T-cell differentiation protein	-1,67	0,00290473
Tspan1	tetraspanin 1	-1,67	0,00082139
Aldob	aldolase B, fructose-bisphosphate	-1,67	0,00241812
Fitm1	fat storage-inducing transmembrane protein 1	-1,67	0,00038601
Kcnn2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	-1,67	0,00066358
Slc22a2	solute carrier family 22 (organic cation transporter), member 2	-1,67	0,00078398
Dpep1	dipeptidase 1 (renal)	-1,67	0,00048015

Aadac	arylacetamide deacetylase (esterase)	-1,67	0,00017005
B830017H08Rik	RIKEN cDNA B830017H08 gene	-1,67	0,00026470
Cyp2d26	cytochrome P450, family 2, subfamily d, polypeptide 26	-1,67	0,00010853
Mgst3	microsomal glutathione S-transferase 3	-1,64	0,00942431
Pcca	propionyl-Coenzyme A carboxylase, alpha polypeptide	-1,64	0,00535324
Scnn1a	sodium channel, nonvoltage-gated 1 alpha	-1,64	0,00547112
Lrrc58	leucine rich repeat containing 58	-1,64	0,00380238
Gchfr	GTP cyclohydrolase I feedback regulator	-1,64	0,00130043
Galnt14	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14	-1,64	0,00061836
Trim63	tripartite motif-containing 63	-1,64	0,00201115
Ndufs2	NADH dehydrogenase (ubiquinone) Fe-S protein 2	-1,64	0,00152016
Suclg1	succinate-CoA ligase, GDP-forming, alpha subunit	-1,64	0,00119485
Ndufab1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	-1,64	0,00062359
Sucnr1	succinate receptor 1	-1,64	0,00076425
A1cf	APOBEC1 complementation factor	-1,64	0,00003200
Cab39l	calcium binding protein 39-like	-1,64	0,00039268
Abhd14a	abhydrolase domain containing 14A	-1,64	0,00011949
Tmem27	transmembrane protein 27	-1,64	0,00005060
Gcnt1	glucosaminyl (N-acetyl) transferase 1, core 2	-1,64	0,00000930
LOC102634389	uncharacterized LOC102634389	-1,64	0,00485000
CYP2F1	cytochrome P450, family 2, subfamily F, polypeptide 1	-1,64	0,00203000
KIAA1191	KIAA1191	-1,64	0,00023900
Gm4013	predicted gene 4013	-1,61	0,00063000
COLGALT2	collagen beta(1-O)galactosyltransferase 2	-1,61	0,00130000
Aldh9a1	aldehyde dehydrogenase 9, subfamily A1	-1,61	0,00702787
Klf9	Kruppel-like factor 9	-1,61	0,00738868
Sfxn2	sideroflexin 2	-1,61	0,00685846
Tmem37	transmembrane protein 37	-1,61	0,00708295
Npb	neuropeptide B	-1,61	0,00531441
Lrba	LPS-responsive beige-like anchor	-1,61	0,00464128
Spag5	sperm associated antigen 5	-1,61	0,00388354
Cdkal1	CDK5 regulatory subunit associated protein 1-like 1	-1,61	0,00205178
Iqgap2	IQ motif containing GTPase activating protein 2	-1,61	0,00114383
Ect2	ect2 oncogene	-1,61	0,00078848
Clybl	citrate lyase beta like	-1,61	0,00029066
Snhg11	small nucleolar RNA host gene 11 (non-protein coding)	-1,61	0,00039544
Nnt	nicotinamide nucleotide transhydrogenase	-1,61	0,00017317
Aqp11	aquaporin 11	-1,61	0,00013192
Cyc1	cytochrome c-1	-1,61	0,00013885

Tfpi2	tissue factor pathway inhibitor 2	-1,59	0,00725041
Nudt4	nudix (nucleoside diphosphate linked moiety X)-type motif 4	-1,59	0,00387031
Idh3a	isocitrate dehydrogenase 3 (NAD+) alpha	-1,59	0,00597849
Ttc32	tetratricopeptide repeat domain 32	-1,59	0,00589992
Tmem116	transmembrane protein 116	-1,59	0,00298010
BC026585	cDNA sequence BC026585	-1,59	0,00424543
Slc6a20b	solute carrier family 6 (neurotransmitter transporter), member 20B	-1,59	0,00226240
Iqcg	IQ motif containing G	-1,59	0,00167877
Med14	mediator complex subunit 14	-1,59	0,00086897
Ndufs8	NADH dehydrogenase (ubiquinone) Fe-S protein 8	-1,59	0,00248945
Oxct1	3-oxoacid CoA transferase 1	-1,59	0,00115044
Slc16a9	solute carrier family 16 (monocarboxylic acid transporters), member 9	-1,59	0,00273549
Vwce	von Willebrand factor C and EGF domains	-1,59	0,00294521
Vpreb1	pre-B lymphocyte gene 1	-1,59	0,00069106
Slc4a4	solute carrier family 4 (anion exchanger), member 4	-1,59	0,00108217
Fars2	phenylalanine-tRNA synthetase 2 (mitochondrial)	-1,59	0,00071588
Aoah	acyloxyacyl hydrolase	-1,59	0,00024277
Dlat	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	-1,59	0,00003280
SLC51B	solute carrier family 51, beta subunit	-1,59	0,00702000
Gapdh-ps14	glyceraldehyde-3-phosphate dehydrogenase, pseudogene 14	-1,59	0,00072800
HIST2H2AA3/HIST2H2AA4	histone cluster 2, H2aa3	-1,59	0,00057800
Ifi44	interferon-induced protein 44	-1,56	0,00607329
Sar1b	SAR1 gene homolog B (S, cerevisiae)	-1,56	0,00518175
Esd	esterase D/formylglutathione hydrolase	-1,56	0,00293989
Slc22a23	solute carrier family 22, member 23	-1,56	0,00117172
Cryl1	crystallin, lambda 1	-1,56	0,00211500
Osbpl1a	oxysterol binding protein-like 1A	-1,56	0,00070092
Amacr	alpha-methylacyl-CoA racemase	-1,56	0,00026397
Bmp7	bone morphogenetic protein 7	-1,56	0,00011306
Agps	alkylglycerone phosphate synthase	-1,56	0,00000856
HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	-1,56	0,00349000
FUOM	fucose mutarotase	-1,56	0,00220000
SUGCT	succinyl-CoA:glutarate-CoA transferase	-1,56	0,00092800
TTC7A	tetratricopeptide repeat domain 7A	-1,56	0,00017300
PLPPR4	phospholipid phosphatase related 4	-1,56	0,00074700
NXPE3	neurexophilin and PC-esterase domain family, member 3	-1,56	0,00054100
Cyp2d22	cytochrome P450, family 2, subfamily d, polypeptide 22	-1,54	0,00444173
Depdc1b	DEP domain containing 1B	-1,54	0,00678738
Hspa8	heat shock protein 8	-1,54	0,00488649

Mrpl46	mitochondrial ribosomal protein L46	-1,54	0,00668229
Ptgr2	prostaglandin reductase 2	-1,54	0,00331605
Acadvl	acyl-Coenzyme A dehydrogenase, very long chain	-1,54	0,00420651
Pla2g5	phospholipase A2, group V	-1,54	0,00154006
Nus1	nuclear undecaprenyl pyrophosphate synthase 1 homolog (S, cerevisiae)	-1,54	0,00130058
Ak7	adenylate kinase 7	-1,54	0,00085965
Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	-1,54	0,00281588
Glyat	glycine-N-acyltransferase	-1,54	0,00201235
Bpnt1	bisphosphate 3'-nucleotidase 1	-1,54	0,00168723
Slc25a39	solute carrier family 25, member 39	-1,54	0,00193822
Iah1	isoamyl acetate-hydrolyzing esterase 1 homolog (S, cerevisiae)	-1,54	0,00122843
Atp6v1a	ATPase, H+ transporting, lysosomal V1 subunit A	-1,54	0,00076280
Slc23a3	solute carrier family 23 (nucleobase transporters), member 3	-1,54	0,00005470
Gjb2	gap junction protein, beta 2	-1,54	0,00036640
Slc25a17	solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17	-1,54	0,00029170
Sfxn1	sideroflexin 1	-1,54	0,00025563
Rb1	retinoblastoma 1	-1,54	0,00002730
Uqcrfs1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	-1,54	0,00002390
Adk	adenosine kinase	-1,54	0,00000995
Cisd1	CDGSH iron sulfur domain 1	-1,54	0,00000259
TMEM229A	transmembrane protein 229A	-1,54	0,00197000
4930481A15Rik	RIKEN cDNA 4930481A15 gene	-1,54	0,00048100
MAP7	microtubule-associated protein 7	-1,54	0,00035500
DNAH12	dynein, axonemal, heavy chain 12	-1,54	0,00015200
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	-1,39	0,00140315
Up-regulated			
Cxcl2	chemokine (C-X-C motif) ligand 2	1,32	0,00395815
Actb	actin, beta	1,35	0,00997899
Dtx3	deltex 3 homolog (Drosophila)	1,51	0,00184538
PEA15	phosphoprotein enriched in astrocytes 15	1,51	0,00119000
Spon1	spondin 1, (f-spondin) extracellular matrix protein	1,51	0,00002450
TENM3	teneurin transmembrane protein 3	1,52	0,00746000
Thsd1	thrombospondin, type I, domain 1	1,52	0,00433737
POU2F2	POU class 2 homeobox 2	1,52	0,00270000
CCDC184	coiled-coil domain containing 184	1,52	0,00315000
Cd200r1	CD200 receptor 1	1,52	0,00135133
Crym	crystallin, mu	1,52	0,00226392
Zfp740	zinc finger protein 740	1,52	0,00037731

Tmeff1	transmembrane protein with EGF-like and two follistatin-like domains 1	1,52	0,00027038
Inhbb	inhibin beta-B	1,53	0,00524078
B2m	beta-2 microglobulin	1,53	0,00265061
1600029O15Rik	RIKEN cDNA 1600029O15 gene	1,53	0,00094096
MFSD2A	major facilitator superfamily domain containing 2A	1,53	0,00234000
2310035P21Rik	RIKEN cDNA 2310035P21 gene	1,53	0,00008070
Postn	periostin, osteoblast specific factor	1,53	0,00027639
Procr	protein C receptor, endothelial	1,54	0,00700356
Tnip2	TNFAIP3 interacting protein 2	1,54	0,00690884
Kcnk3	potassium channel, subfamily K, member 3	1,54	0,00569228
Tro	trophinin	1,54	0,00481998
Col23a1	collagen, type XXIII, alpha 1	1,54	0,00181177
Ifngr1	interferon gamma receptor 1	1,54	0,00140742
Gsdmd	gasdermin D	1,54	0,00037279
MAP7D1	MAP7 domain containing 1	1,54	0,00034100
PCDHB14	protocadherin beta 14	1,54	0,00005410
Ehbp1l1	EH domain binding protein 1-like 1	1,55	0,00678198
E330032C10Rik	RIKEN cDNA E330032C10 gene	1,55	0,00568000
Fam105a	family with sequence similarity 105, member A	1,55	0,00324852
Synpo	synaptopodin	1,55	0,00473111
FAM3D	family with sequence similarity 3, member D	1,55	0,00195000
Pot1b	protection of telomeres 1B	1,55	0,00132454
BEAN1	brain expressed, associated with NEDD4, 1	1,56	0,00282000
C1qtnf7	C1q and tumor necrosis factor related protein 7	1,56	0,00470089
Ifitm7	interferon induced transmembrane protein 7	1,56	0,00455554
C1qb	complement component 1, q subcomponent, beta polypeptide	1,56	0,00256316
Arid5a	AT rich interactive domain 5A (MRF1-like)	1,56	0,00105630
Itgb4	integrin beta 4	1,56	0,00001760
Nav1	neuron navigator 1	1,57	0,00163736
Gpm6b	glycoprotein m6b	1,57	0,00330637
C7	complement component 7	1,57	0,00078200
Gria4	glutamate receptor, ionotropic, AMPA4 (alpha 4)	1,57	0,00087762
Runx2	runt related transcription factor 2	1,57	0,00108565
Hs6st2	heparan sulfate 6-O-sulfotransferase 2	1,57	0,00151680
Lmod1	leiomodin 1 (smooth muscle)	1,57	0,00148332
Myh9	myosin, heavy polypeptide 9, non-muscle	1,57	0,00002880
PLEC	plectin	1,57	0,00001490
Duxbl1 (includes others)	double homeobox B-like 1	1,58	0,00498000
Zyx	zyxin	1,58	0,00686626

Pdgfra	platelet derived growth factor receptor, alpha polypeptide	1,58	0,00241345
Mmp7	matrix metallopeptidase 7	1,58	0,00284682
Plekhg2	pleckstrin homology domain containing, family G (with RhoGef domain) member 2	1,58	0,00127753
Hbegf	heparin-binding EGF-like growth factor	1,58	0,00011449
Rras	Harvey rat sarcoma oncogene, subgroup R	1,58	0,00000544
Mmp2	matrix metallopeptidase 2	1,59	0,00724283
Slamf9	SLAM family member 9	1,59	0,00685326
Hmcn1	hemicentin 1	1,59	0,00380406
KIAA0895L	KIAA0895-like	1,59	0,00611000
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	1,59	0,00177273
Il6st	interleukin 6 signal transducer	1,59	0,00005560
Gm29790	predicted gene, 29790	1,60	0,00484000
Cntn6	contactin 6	1,60	0,00126061
Gng8	guanine nucleotide binding protein (G protein), gamma 8	1,60	0,00123813
Myadm	myeloid-associated differentiation marker	1,60	0,00079076
S1pr2	sphingosine-1-phosphate receptor 2	1,60	0,00054251
Cbarp	calcium channel, voltage-dependent, beta subunit associated regulatory protein	1,60	0,00006380
Tc2n	tandem C2 domains, nuclear	1,61	0,00714254
Inpp5d	inositol polyphosphate-5-phosphatase D	1,61	0,00629272
Camk2b	calcium/calmodulin-dependent protein kinase II, beta	1,61	0,00576891
Adh7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	1,61	0,00489196
Tlr1	toll-like receptor 1	1,61	0,00192742
Tgfb1	transforming growth factor, beta 1	1,61	0,00185473
Crip1	cysteine-rich protein 1 (intestinal)	1,61	0,00098435
Stat4	signal transducer and activator of transcription 4	1,62	0,00502516
Zc3h6	zinc finger CCCH type containing 6	1,62	0,00448020
Gpsm3	G-protein signalling modulator 3 (AGS3-like, C, elegans)	1,62	0,00641185
ADGRA2	adhesion G protein-coupled receptor A2	1,62	0,00554000
Efs	embryonal Fyn-associated substrate	1,62	0,00416027
Fkbp10	FK506 binding protein 10	1,62	0,00378981
Meis3	Meis homeobox 3	1,62	0,00184210
Muc4	mucin 4	1,62	0,00297751
Nkain4	Na+/K+ transporting ATPase interacting 4	1,62	0,00174388
Sparc	secreted acidic cysteine rich glycoprotein	1,62	0,00060352
Col12a1	collagen, type XII, alpha 1	1,62	0,00000021
Sorbs2	sorbin and SH3 domain containing 2	1,63	0,00062293
Unc5a	unc-5 homolog A (C, elegans)	1,63	0,00209716
Ugcg	UDP-glucose ceramide glucosyltransferase	1,63	0,00133485
Tnnt2	troponin T2, cardiac	1,64	0,00328438

Lox	lysyl oxidase	1,64	0,00518796
Stx1a	syntaxin 1A (brain)	1,65	0,00320208
Lyl1	lymphoblastomic leukemia 1	1,65	0,00424853
Slc11a1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1,65	0,00372550
Mmrn1	multimerin 1	1,65	0,00302821
Crlf2	cytokine receptor-like factor 2	1,65	0,00289161
Bok	BCL2-related ovarian killer protein	1,65	0,00028961
Rtn4	reticulon 4	1,66	0,00461938
Pamr1	peptidase domain containing associated with muscle regeneration 1	1,66	0,00389178
Tmem158	transmembrane protein 158	1,66	0,00406425
Pik3r5	phosphoinositide-3-kinase, regulatory subunit 5, p101	1,66	0,00282719
Gdf9	growth differentiation factor 9	1,66	0,00125671
Pdlim1	PDZ and LIM domain 1 (elfin)	1,66	0,00048683
C1orf198	chromosome 1 open reading frame 198	1,67	0,00739000
Col2a1	collagen, type II, alpha 1	1,67	0,00526899
Dock10	dedicator of cytokinesis 10	1,67	0,00117190
Synj2	synaptojanin 2	1,67	0,00072451
Adamtsl5	ADAMTS-like 5	1,67	0,00001990
Cx3cr1	chemokine (C-X3-C) receptor 1	1,68	0,00480877
Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide	1,68	0,00443714
Hivep3	human immunodeficiency virus type I enhancer binding protein 3	1,68	0,00605928
Tnik	TRAF2 and NCK interacting kinase	1,68	0,00217911
Egflam	EGF-like, fibronectin type III and laminin G domains	1,68	0,00069338
Hcls1	hematopoietic cell specific Lyn substrate 1	1,68	0,00053185
MKRN2OS	MKRN2 opposite strand	1,68	0,00011700
Stra6l	STRA6-like	1,69	0,00355000
Col5a2	collagen, type V, alpha 2	1,69	0,00313506
Col15a1	collagen, type XV, alpha 1	1,69	0,00029020
Ptrf	polymerase I and transcript release factor	1,69	0,00097095
Rbp1	retinol binding protein 1, cellular	1,69	0,00032857
Irak3	interleukin-1 receptor-associated kinase 3	1,69	0,00000927
Lpxn	leupaxin	1,70	0,00545926
ZNF616	zinc finger protein 616	1,70	0,00386000
Ciita	class II transactivator	1,70	0,00091141
Frmd3	FERM domain containing 3	1,70	0,00052296
Vim	vimentin	1,70	0,00051564
Esyt1	extended synaptotagmin-like protein 1	1,70	0,00001590
Cd300lf	CD300 antigen like family member F	1,71	0,00359009
Rasgef1a	RasGEF domain family, member 1A	1,71	0,00402791

Igf2bp2	insulin-like growth factor 2 mRNA binding protein 2	1,71	0,00054944
Lmna	lamin A	1,71	0,00216762
P3H3	prolyl 3-hydroxylase 3	1,71	0,00178000
Antxr1	anthrax toxin receptor 1	1,72	0,00440600
Rhoc	ras homolog gene family, member C	1,72	0,00481923
Garnl3	GTPase activating RANGAP domain-like 3	1,72	0,00159376
A730008I21Rik	RIKEN cDNA A730008I21 gene	1,73	0,00342284
Nek6	NIMA (never in mitosis gene a)-related expressed kinase 6	1,73	0,00015588
Dst	dystonin	1,74	0,00696501
PAK3	p21 protein (Cdc42/Rac)-activated kinase 3	1,74	0,00530000
C1orf100	chromosome 1 open reading frame 100	1,74	0,00273000
Lair1	leukocyte-associated Ig-like receptor 1	1,74	0,00238892
Olfml2b	olfactomedin-like 2B	1,74	0,00227414
Csf1	colony stimulating factor 1 (macrophage)	1,74	0,00099039
Pltp	phospholipid transfer protein	1,74	0,00110555
Arpc1b	actin related protein 2/3 complex, subunit 1B	1,74	0,00054525
Cd248	CD248 antigen, endosialin	1,74	0,00045621
Timp2	tissue inhibitor of metalloproteinase 2	1,74	0,00038810
Emp3	epithelial membrane protein 3	1,74	0,00000156
CLEC10A	C-type lectin domain family 10, member A	1,75	0,00515000
Csdc2	cold shock domain containing C2, RNA binding	1,75	0,00379612
Cxcl13	chemokine (C-X-C motif) ligand 13	1,75	0,00052198
Gm39680	predicted gene, 39680	1,75	0,00000818
Actc1	actin, alpha, cardiac muscle 1	1,76	0,00172284
Fbn1	fibrillin 1	1,76	0,00128103
Sepn1	selenoprotein N, 1	1,77	0,00608359
Wbscr17	Williams-Beuren syndrome chromosome region 17 homolog (human)	1,77	0,00578499
Ddr2	discoidin domain receptor family, member 2	1,78	0,00149479
Wt1	Wilms tumor 1 homolog	1,79	0,00263757
Pcdh10	protocadherin 10	1,79	0,00222523
Dpysl4	dihydropyrimidinase-like 4	1,79	0,00059011
Cybb	cytochrome b-245, beta polypeptide	1,80	0,00111453
Gm33298	predicted gene, 33298	1,80	0,00148000
Efemp2	epidermal growth factor-containing fibulin-like extracellular matrix protein 2	1,80	0,00009270
Stat3	signal transducer and activator of transcription 3	1,80	0,00007710
Chst12	carbohydrate sulfotransferase 12	1,81	0,00281312
Lum	lumican	1,81	0,00071985
Ckap4	cytoskeleton-associated protein 4	1,81	0,00159650
Krt8	keratin 8	1,81	0,00000212

Tlr2	toll-like receptor 2	1,83	0,00710037
Nfkb2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	1,84	0,00550569
Pglyrp1	peptidoglycan recognition protein 1	1,84	0,00271478
Ccl11	chemokine (C-C motif) ligand 11	1,84	0,00330891
Plek	pleckstrin	1,84	0,00219709
LOC728392	uncharacterized LOC728392	1,84	0,00178000
MMP23B	matrix metallopeptidase 23B	1,84	0,00009830
Mfi2	antigen p97 (melanoma associated) identified by monoclonal antibodies 133,2 and 96,5	1,85	0,00492744
Mfap2	microfibrillar-associated protein 2	1,85	0,00268228
Col6a2	collagen, type VI, alpha 2	1,85	0,00050407
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	1,85	0,00053480
Myof	myoferlin	1,85	0,00024512
Areg	amphiregulin	1,85	0,00007950
Akap12	A kinase (PRKA) anchor protein (gravin) 12	1,85	0,00004360
Irf8	interferon regulatory factor 8	1,85	0,00002760
Tmsb4x (includes others)	thymosin, beta 4, X chromosome	1,85	0,00003200
Pcdh9	protocadherin 9	1,86	0,00946012
ZBTB8B	zinc finger and BTB domain containing 8B	1,86	0,00585000
Fam155a	family with sequence similarity 155, member A	1,86	0,00448713
Fyb	FYN binding protein	1,86	0,00453929
Il1b	interleukin 1 beta	1,86	0,00270017
Sdc1	syndecan 1	1,86	0,00033755
Trpv6	transient receptor potential cation channel, subfamily V, member 6	1,86	0,00004920
Fam171b	family with sequence similarity 171, member B	1,87	0,00044044
Tgfb1i1	transforming growth factor beta 1 induced transcript 1	1,87	0,00114505
Hspb1	heat shock protein 1	1,87	0,00096796
Vgll3	vestigial like 3 (Drosophila)	1,87	0,00010022
Vav1	vav 1 oncogene	1,88	0,00499737
Myo1f	myosin IF	1,88	0,00158189
Flna	filamin, alpha	1,88	0,00010633
CXCL6	chemokine (C-X-C motif) ligand 6	1,90	0,00457000
Gm5067	predicted gene 5067	1,90	0,00528791
Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1,90	0,00433846
Fap	fibroblast activation protein	1,90	0,00047633
Tuba1a	tubulin, alpha 1A	1,90	0,00074464
Ifi27l2a/Ifi27l2b	interferon, alpha-inducible protein 27 like 2A	1,91	0,00209000
Dnm3os	dynamin 3, opposite strand	1,91	0,00173453
Tmem173	transmembrane protein 173	1,91	0,00185531
Lbh	limb-bud and heart	1,91	0,00013459

Ccl20	chemokine (C-C motif) ligand 20	1,92	0,00757383
Slc15a3	solute carrier family 15, member 3	1,92	0,00491781
CLEC4A	C-type lectin domain family 4, member A	1,92	0,00157000
Mst1r	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	1,92	0,00044329
Vwc2	von Willebrand factor C domain containing 2	1,93	0,00404572
Col6a1	collagen, type VI, alpha 1	1,93	0,00519340
Ptch2	patched homolog 2	1,93	0,00325673
Hpgds	hematopoietic prostaglandin D synthase	1,93	0,00087700
Vipr2	vasoactive intestinal peptide receptor 2	1,93	0,00105498
Thbs3	thrombospondin 3	1,93	0,00130123
Bcl2a1c	B-cell leukemia/lymphoma 2 related protein A1c	1,94	0,00758314
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	1,94	0,00480000
SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1,94	0,00446000
2310043M15Rik	RIKEN cDNA 2310043M15 gene	1,94	0,00324145
Lrp1	low density lipoprotein receptor-related protein 1	1,94	0,00066749
Tpm1	tropomyosin 1, alpha	1,94	0,00000868
AW112010	expressed sequence AW112010	1,95	0,00411484
Edn1	endothelin 1	1,95	0,00122156
F3	coagulation factor III	1,96	0,00470628
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	1,96	0,00341000
C1qtnf5	C1q and tumor necrosis factor related protein 5	1,96	0,00380955
Arsj	arylsulfatase J	1,96	0,00077534
4930588G05Rik	RIKEN cDNA 4930588G05 gene	1,96	0,00209884
Ncf1	neutrophil cytosolic factor 1	1,96	0,00038228
Il1r2	interleukin 1 receptor, type II	1,96	0,00003490
Lrrc25	leucine rich repeat containing 25	1,97	0,00130160
Col16a1	collagen, type XVI, alpha 1	1,97	0,00033561
Shisa3	shisa homolog 3 (Xenopus laevis)	1,98	0,00441042
Pf4	platelet factor 4	1,98	0,00661321
Ankrd1	ankyrin repeat domain 1 (cardiac muscle)	1,98	0,00079822
Thbs2	thrombospondin 2	1,98	0,00058586
Glipr1	GLI pathogenesis-related 1 (glioma)	1,98	0,00005260
Kif1a	kinesin family member 1A	1,99	0,00425129
Jun	Jun oncogene	1,99	0,00000003
Hk2	hexokinase 2	2,00	0,00214794
Apobec1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	2,00	0,00152598
Gpnmb	glycoprotein (transmembrane) nmb	2,01	0,00268795
Clec5a	C-type lectin domain family 5, member a	2,01	0,00115478
Fign	fidgetin	2,02	0,00826869

2210016H18Rik	RIKEN cDNA 2210016H18 gene	2,02	0,00654241
Wnt4	wingless-related MMTV integration site 4	2,02	0,00000164
Clec7a	C-type lectin domain family 7, member a	2,03	0,00359371
Eno2	enolase 2, gamma neuronal	2,03	0,00325402
Csf2rb	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	2,04	0,00348451
Nudt6	nudix (nucleoside diphosphate linked moiety X)-type motif 6	2,04	0,00159545
Itga4	integrin alpha 4	2,05	0,00226084
Trbv1	T cell receptor beta, variable 1	2,05	0,00227000
Col1a2	collagen, type I, alpha 2	2,05	0,00000769
Twist2	twist homolog 2 (Drosophila)	2,06	0,00208382
CSTA	cystatin A (stefin A)	2,07	0,00550000
Efhd2	EF hand domain containing 2	2,07	0,00000000
Socs2	suppressor of cytokine signaling 2	2,08	0,00125522
Laptm5	lysosomal-associated protein transmembrane 5	2,08	0,00136304
Inhba	inhibin beta-A	2,08	0,00013431
Lsp1	lymphocyte specific 1	2,08	0,00011626
Birc3	baculoviral IAP repeat-containing 3	2,09	0,00483301
Relb	avian reticuloendotheliosis viral (v-rel) oncogene related B	2,09	0,00665853
Psmb8	proteasome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase 7)	2,09	0,00137712
Serping1	serine (or cysteine) peptidase inhibitor, clade G, member 1	2,09	0,00022201
Tnni2	troponin I, skeletal, fast 2	2,09	0,00002140
Cyth4	cytohesin 4	2,11	0,00154584
Cpne7	copine VII	2,12	0,00370499
ADGRE1	adhesion G protein-coupled receptor E1	2,14	0,00285000
Adamts2	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2	2,16	0,00548476
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	2,16	0,00070706
Acta1	actin, alpha 1, skeletal muscle	2,16	0,00006830
Mirg	miRNA containing gene	2,16	0,00010286
Ccl9	chemokine (C-C motif) ligand 9	2,16	0,00005090
Sox9	SRY-box containing gene 9	2,18	0,00559392
Sntg2	syntrophin, gamma 2	2,18	0,00072400
C1s	complement component 1, s subcomponent	2,19	0,00023879
Ccdc80	coiled-coil domain containing 80	2,19	0,00003740
Klf6	Kruppel-like factor 6	2,20	0,00283277
Mrc1	mannose receptor, C type 1	2,21	0,00110458
Myocd	myocardin	2,21	0,00074337
Parp3	poly (ADP-ribose) polymerase family, member 3	2,21	0,00006810
CLEC4C	C-type lectin domain family 4, member C	2,22	0,00699000

Ms4a4b (includes others)	membrane-spanning 4-domains, subfamily A, member 4B	2,22	0,00572000
Gdf10	growth differentiation factor 10	2,24	0,00138785
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	2,25	0,00642459
Trim29	tripartite motif-containing 29	2,26	0,00576890
C1qa	complement component 1, q subcomponent, alpha polypeptide	2,26	0,00328903
Meg3	maternally expressed 3	2,26	0,00002080
Rhou	ras homolog gene family, member U	2,29	0,00138383
Serpinb5	serine (or cysteine) peptidase inhibitor, clade B, member 5	2,29	0,00297478
Creb5	cAMP responsive element binding protein 5	2,30	0,00512008
Il34	interleukin 34	2,30	0,00126166
Cyr61	cysteine rich protein 61	2,30	0,00048726
Phlda3	pleckstrin homology-like domain, family A, member 3	2,30	0,00006450
Acta2	actin, alpha 2, smooth muscle, aorta	2,30	0,00000188
Ubd	ubiquitin D	2,31	0,00073263
Tubb6	tubulin, beta 6	2,31	0,00000675
Ndp	Norrie disease (pseudoglioma) (human)	2,34	0,00230022
Cx3cl1	chemokine (C-X3-C motif) ligand 1	2,34	0,00001120
Ednra	endothelin receptor type A	2,35	0,00344960
Ccl19	chemokine (C-C motif) ligand 19	2,35	0,00124702
Clca3a1/Clca3a2	chloride channel accessory 3A1	2,35	0,00000836
Foxj1	forkhead box J1	2,36	0,00276918
Dpysl3	dihydropyrimidinase-like 3	2,36	0,00142205
Fmo5	flavin containing monooxygenase 5	2,36	0,00048053
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	2,36	0,00028100
Ccl17	chemokine (C-C motif) ligand 17	2,37	0,00465528
Spon2	spondin 2, extracellular matrix protein	2,37	0,00098260
Ccdc88b	coiled-coil domain containing 88B	2,38	0,00590947
Iqsec3	IQ motif and Sec7 domain 3	2,38	0,00254086
Cd14	CD14 antigen	2,38	0,00002010
Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	2,39	0,00167531
HLA-DRB5	major histocompatibility complex, class II, DR beta 5	2,41	0,00499000
Dtx4	deltex 4 homolog (Drosophila)	2,41	0,00122821
Plac8	placenta-specific 8	2,41	0,00092193
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	2,41	0,00025483
Ms4a7	membrane-spanning 4-domains, subfamily A, member 7	2,44	0,00156870
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	2,44	0,00109000
Pdlim7	PDZ and LIM domain 7	2,44	0,00005310
Cpxm2	carboxypeptidase X 2 (M14 family)	2,45	0,00000363
Scara3	scavenger receptor class A, member 3	2,46	0,00089339

Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	2,47	0,00012336
2200002D01Rik	RIKEN cDNA 2200002D01 gene	2,47	0,00005410
Socs3	suppressor of cytokine signaling 3	2,48	0,00430235
Fbln1	fibulin 1	2,48	0,00340385
Fcrls	Fc receptor-like S, scavenger receptor	2,48	0,00055090
Wdfy4	WD repeat and FYVE domain containing 4	2,49	0,00641305
Icam1	intercellular adhesion molecule 1	2,50	0,00013089
Cygb	cytoglobin	2,51	0,00212215
Spp1	secreted phosphoprotein 1	2,52	0,00005040
Col1a1	collagen, type I, alpha 1	2,53	0,00033859
Loxl1	lysyl oxidase-like 1	2,54	0,00082523
Tpm2	tropomyosin 2, beta	2,54	0,00074746
Anxa13	annexin A13	2,55	0,00254006
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	2,57	0,00232000
CCL21	chemokine (C-C motif) ligand 21	2,58	0,00065500
Coro1a	coronin, actin binding protein 1A	2,58	0,00011610
GXYLT2	glucoside xylosyltransferase 2	2,59	0,00086900
ANXA8/ANXA8L1	annexin A8-like 1	2,59	0,00005990
Fos	FBJ osteosarcoma oncogene	2,59	0,00000881
Nckap1l	NCK associated protein 1 like	2,60	0,00116803
Runx1	runt related transcription factor 1	2,60	0,00079936
Mfap4	microfibrillar-associated protein 4	2,62	0,00694925
Atf3	activating transcription factor 3	2,62	0,00000046
Tnfsf13b	tumor necrosis factor (ligand) superfamily, member 13b	2,63	0,00285740
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	2,66	0,00126704
Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	2,69	0,00078037
Timp1	tissue inhibitor of metalloproteinase 1	2,74	0,00023804
Penk	preproenkephalin	2,75	0,00129473
Upk3a	uroplakin 3A	2,76	0,00342461
Lgi2	leucine-rich repeat LGI family, member 2	2,77	0,00110409
Uchl1	ubiquitin carboxy-terminal hydrolase L1	2,77	0,00000199
Tagln	transgelin	2,78	0,00009010
Lcn2	lipocalin 2	2,79	0,00000929
Krt18	keratin 18	2,79	0,00000089
Clu	clusterin	2,81	0,00000199
Adam23	a disintegrin and metallopeptidase domain 23	2,82	0,00274975
Cp	ceruloplasmin	2,83	0,00036127
Apbb1ip	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	2,83	0,00017779
Aif1	allograft inflammatory factor 1	2,84	0,00428890

Aldh3b2	aldehyde dehydrogenase 3 family, member B2	2,84	0,00279623
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2,84	0,00278000
Il33	interleukin 33	2,89	0,00129850
Junb	Jun-B oncogene	2,89	0,00023563
Ptn	pleiotrophin	2,90	0,00027148
C1qc	complement component 1, q subcomponent, C chain	2,91	0,00020037
Gjb6	gap junction protein, beta 6	2,91	0,00018117
Il18r1	interleukin 18 receptor 1	2,92	0,00007490
Adamts19	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19	2,97	0,00056703
Mfap5	microfibrillar associated protein 5	2,99	0,00215019
Slit3	slit homolog 3 (Drosophila)	3,00	0,00083177
Hp	haptoglobin	3,00	0,00005760
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	3,02	0,00078700
Pdlim3	PDZ and LIM domain 3	3,02	0,00062099
Lrrn2	leucine rich repeat protein 2, neuronal	3,02	0,00005380
Tgfbi	transforming growth factor, beta induced	3,11	0,00038365
Thy1	thymus cell antigen 1, theta	3,12	0,00036506
Mgp	matrix Gla protein	3,25	0,00001510
S100a6	S100 calcium binding protein A6 (calcyclin)	3,28	0,00000094
Ccl7	chemokine (C-C motif) ligand 7	3,29	0,00025846
Trem2	triggering receptor expressed on myeloid cells 2	3,53	0,00130911
Cd44	CD44 antigen	3,56	0,00148334
Ltbp2	latent transforming growth factor beta binding protein 2	3,58	0,00036313
RAB7B	RAB7B, member RAS oncogene family	3,59	0,00066900
Vcam1	vascular cell adhesion molecule 1	3,66	0,00225302
BCL2A1	BCL2-related protein A1	3,67	0,00234000
Cyp2j13	cytochrome P450, family 2, subfamily j, polypeptide 13	3,73	0,00206565
Sulf1	sulfatase 1	3,80	0,00053808
Serpina3g (includes others)	serine (or cysteine) peptidase inhibitor, clade A, member 3G	3,81	0,00035700
Adcy7	adenylate cyclase 7	3,85	0,00018827
Osmr	oncostatin M receptor	3,91	0,00066072
Ctss	cathepsin S	3,92	0,00141361
Upk1b	uroplakin 1B	3,92	0,00020614
Ccl5	chemokine (C-C motif) ligand 5	3,92	0,00000733
Sprr2f	small proline-rich protein 2F	4,04	0,00237408
Egr1	early growth response 1	4,06	0,00000855
Krt19	keratin 19	4,08	0,00004330
LYZ	lysozyme	4,10	0,00028000
Cnn1	calponin 1	4,17	0,00000077

E330037M01Rik	RIKEN cDNA E330037M01 gene	4,33	0,00278427
Pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	4,74	0,00014600
Sprr1a	small proline-rich protein 1A	4,87	0,00012525
Fam25c	family with sequence similarity 25, member C	4,96	0,00041500
Egr2	early growth response 2	5,02	0,00000933
Ccl2	chemokine (C-C motif) ligand 2	5,28	0,00028207
AOC1	amine oxidase, copper containing 1	5,32	0,00038400
Cxcl10	chemokine (C-X-C motif) ligand 10	6,99	0,00071279
Krt20	keratin 20	9,35	0,00000438
C3	complement component 3	12,24	0,00005770

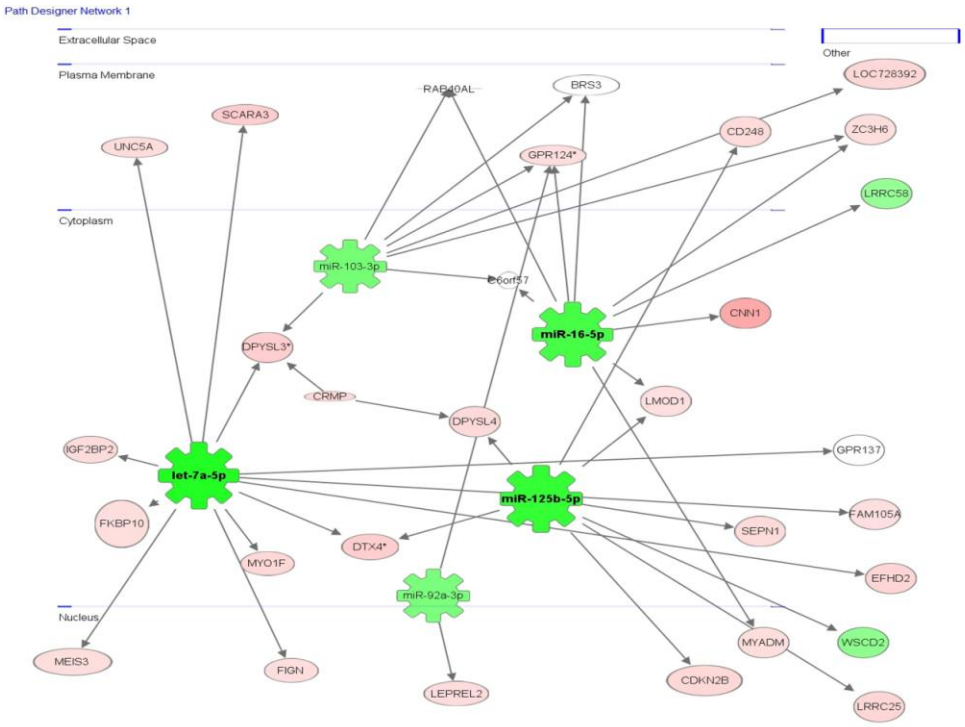
Additional Table 4: Common deregulated miRNAs between human UPJ (urine) and mouse partial UUO (kidney tissue).

	Human UPJ		Mouse partial UUO	
Symbol	**Exp Fold Change**	**Exp p-value**	**Exp Fold Change**	**Exp p-value**
let-7a-5p	-1,559	3,17E-03	-3,558	3,93E-03
miR-125b-5p	-1,18	3,03E-02	-3,219	3,15E-03
miR-26a-5p	-1,376	3,03E-02	-3,175	1,13E-03
miR-16-5p	-1,293	2,27E-03	-2,913	6,97E-04
miR-29b-3p	-1,153	2,93E-02	10,073	3,10E-08
miR-19b-3p	-1,188	4,13E-02	-2,448	1,89E-03
miR-103-3p	-1,135	6,67E-03	-2,179	6,54E-03
miR-151-5p	-1,162	9,09E-03	-2,144	8,04E-03
miR-92a-3p	-1,185	3,93E-03	-2,076	8,14E-03
miR-455-3p	1,066	4,69E-02	-1,933	1,34E-02
miR-142-3p	-1,116	2,65E-03	-1,93	3,03E-03
miR-365-3p	1,27	2,22E-02	-1,928	1,45E-02
miR-140-5p	-1,1	5,27E-03	-1,69	4,05E-03
miR-193a-3p	1,211	1,42E-02	-1,221	2,70E-03
miR-203a-3p	-1,186	1,17E-02	-1,198	1,21E-02
miR-10a-3p	-1,139	1,10E-02	-1,174	4,67E-03
miR-106b-3p	1,078	4,41E-02	-1,04	8,56E-05
miR-24-1-5p	1,096	8,30E-03	1,05	8,59E-04

Additional Table 5: miRNAs and their respective mRNA targets.

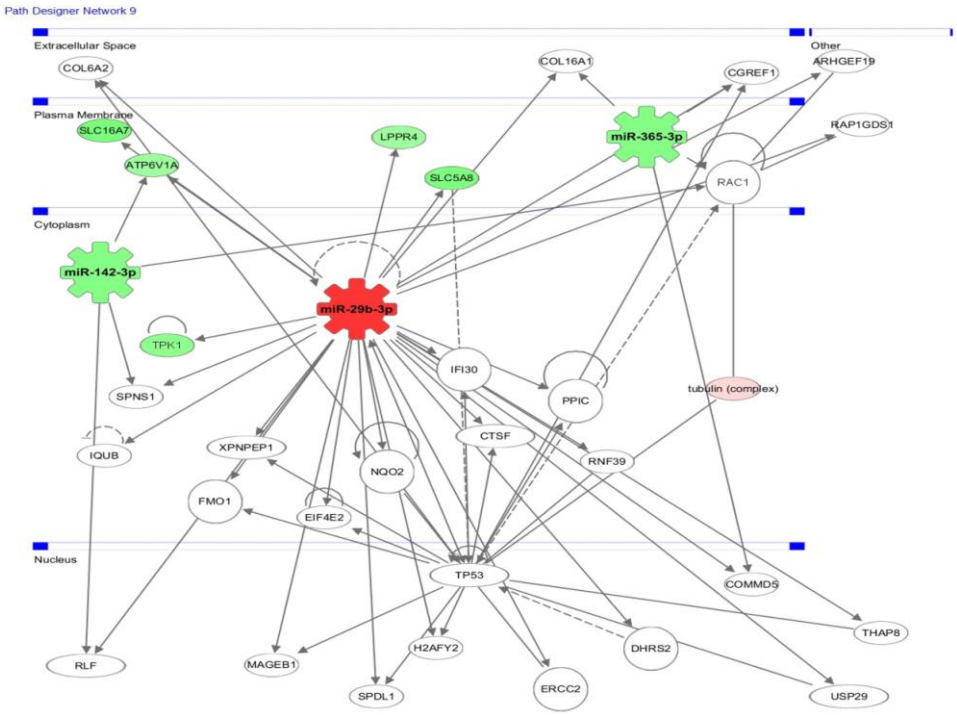
Gene Symbol	Description	Fold Change	p-value	No of miRNAs targeting this mRNA	let-7a-5p	miR-125b-5p	miR-16-5p	miR-26a-5p	miR-29b-3p
Adamts19	**a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19**	**2,97**	**5,67E-04**	**2**				**1**	**1**
Dtx4	**deltex 4 homolog (Drosophila)**	**2,41**	**1,23E-03**	**2**	**1**	**1**			
Lmod1	**leiomodin 1 (smooth muscle)**	**1,57**	**1,48E-03**	**2**		**1**	**1**		
Nav1	**neuron navigator 1**	**1,57**	**1,64E-03**	**2**				**1**	**1**
Atp6v1a	ATPase, H+ transporting, lysosomal V1 subunit A	-1,54	7,63E-04	1					1
C11orf54	chromosome 11 open reading frame 54	-2,27	8,04E-04	1					1
Cd248	CD248 antigen, endosialin	1,74	4,56E-04	1		1			
Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1,90	4,34E-03	1		1			
Cnn1	calponin 1	4,17	7,74E-07	1			1		
Dpysl3	dihydropyrimidinase-like 3	2,36	1,42E-03	1	1				
Dpysl4	dihydropyrimidinase-like 4	1,79	5,90E-04	1		1			
Efhd2	EF hand domain containing 2	2,07	3,45E-09	1	1				
Fam105a	family with sequence similarity 105, member A	1,55	3,25E-03	1	1				
Fign	fidgetin	2,02	8,27E-03	1	1				
Fkbp10	FK506 binding protein 10	1,62	3,79E-03	1	1				
Gcsh	glycine cleavage system protein H (aminomethyl carrier)	-1,75	1,72E-05	1					1
Gpr124	G protein-coupled receptor 124	1,62	5,54E-03	1			1		
Hbegf	heparin-binding EGF-like growth factor	1,58	1,14E-04	1					1
Igf2bp2	insulin-like growth factor 2 mRNA binding protein 2	1,71	5,49E-04	1	1				
KIAA0895L	KIAA0895-like	1,59	6,11E-03	1					1
Lrrc25	leucine rich repeat containing 25	1,97	1,30E-03	1		1			
Lrrc58	leucine rich repeat containing 58	-1,64	3,80E-03	1			1		
Meis3	Meis homeobox 3	1,62	1,84E-03	1	1				
Myadm	myeloid-associated differentiation marker	1,60	7,91E-04	1			1		
Myo1f	myosin IF	1,88	1,58E-03	1	1				
Pcdh9	protocadherin 9	1,86	9,46E-03	1				1	
Rasgef1a	RasGEF domain family, member 1A	1,71	4,03E-03	1				1	
Scara3	scavenger receptor class A, member 3	2,46	8,93E-04	1	1				
Sepn1	selenoprotein N, 1	1,77	6,08E-03	1		1			
Slc16a7	solute carrier family 16 (monocarboxylic acid transporters), member 7	-2,13	5,44E-03	1					1
Slc22a7	solute carrier family 22 (organic anion transporter), member 7	-1,96	3,89E-03	1					1
Slc5a8	solute carrier family 5 (iodide transporter), member 8	-2,04	5,28E-03	1					1
Tpk1	thiamine pyrophosphokinase	-1,79	2,74E-04	1					1
Unc5a	unc-5 homolog A (C, elegans)	1,63	2,10E-03	1	1				
Wscd2	WSC domain containing 2	-1,79	4,49E-03	1		1			
					11	8	5	4	11

Additional file 6: Molecular network predicted by IPA including let-7a-5p, miR-125b-5p and miR-16-5p and possibly related to hematological and immunological disease. Red= up regulated green= down regulated; all colored genes were identified in the partial UUO mouse mRNA dataset.



Additional file 7: Molecular network predicted by IPA including miR-26a-5p and miR-29b-3p and possibly related to organismal Injury and abnormalities. Red= up regulated green= down regulated; all colored genes were identified in the partial UUO mouse mRNA dataset.

Additional file 8: Molecular network predicted by IPA including miR-29b-3p and possibly related to cell death and survival and connective tissue development and function. Red= up regulated green= down regulated; all colored genes were identified in the partial UUO mouse mRNA dataset.



Additional file 9: Sequences of primers used.

Primer sequences for miRNAs were obtained for hsa-let-7a-5p (ref.no. 205727), hsa-miR-125b-5p (ref. no. 205713), hsa-miR-16-5p (ref. no. 205702), hsa-miR-26a-5p (ref.no. 206023) and hsa-miR-29b-3p (ref. no. 204679) from Exiqon (Vedbaek, Denmark).

Primers for mRNAs quantification were obtained from Integrated DNA Technologies (Leuven, Belgium):

mouse DTX4:

m-DTX4-F: ACATCTACTGCCTGGTGGCTATG

m-DTX4-R: GGGCACTGTAAACTCCCATCCTTG

mouse Nav1:

m-Nav1-F: ACCCAAGGGAATGATTCGGTCAG

m-NAV1-R: ACAGCACCGAGCCATGAACATC

mouse LMOD1:

m-LMOD-F: AGATGTCTGTGGATGAAAGCAAGC

m-LMOD1-R: TCCTCTCCATTCTTGGCATCTGTC

mouseADAMTS19:

m-ADAMTS19-F: GCGAGTAGGTGACTGGTCTAAGTG

m-ADAMTS19-R: ACACGAGACTGCATGCCTTTGC

mouse LRRC58:

m-LRRC58-F: TCCAAACCCAAAGTGTGGTGGA

m-LRRC58-R: GGAGAGCACAGATAGTGCATGAGG

mouse-GAPDH:

m-GAPDH-F: CTTTGTCAAGCTCATTTCCTGG

m-GAPDH-R: TCTTGCTCAGTGTCCTTGC

mouse TGFb1:

m-TGFb1-F: CCTGAGTGGCTGTCTTTTGA

m-TGFb1-R: CGTGGAGTTTGTTATCTTTGCTG

7. Conclusions – Outlook

miRNAs have now a place among molecules of great interest to be explored in physiology and disease. The augmentation in the number of reports on miRNAs shows this interest. MiRNAs have been shown to be involved in pathologies as diverse as cancer, neurodegenerative diseases, vascular aging, atherosclerosis and kidney disease. MiRNAs might be useful in explaining (patho)physiological mechanisms, in being potential biomarkers and as therapeutics targets. This wide range of applications, especially as potential biomarkers or therapeutics, derives from their ubiquitous presence and their extreme stability under different biological and storage conditions. Technological solutions (PCR, microarrays, NGS etc.) to study miRNAs are similar to those to study mRNAs and thus have been in place for at least a decade.

7.1 Technological considerations

The observation of the presence of miRNAs in urine has triggered its research in kidney disease and lead to the identification of the number of urinary miRNAs potentially associated to different kidney diseases. Though, there are still obstacles that need to overcome to produce reliable clinically usable results with urinary miRNAs. One of the major problems in miRNA research is the normalization method. Up to now there is no good endogenous control that can be used for normalization. U6, a small non coding RNA was used in most studies in the early years, but recent reports showed its instability between different samples, tissue or biofluids in different species. We observed the same issue for U6 in the CKD progression validation study where the variability of U6 was the highest among the tested endogenous controls. RNU48, another endogenous control, showed a more stable pattern but was not detected in all samples. Fortunately we had developed another approach to select for a stable endogenous control by selecting in the discovery cohort the miRNA with the highest frequency (in all 70 samples) and displaying highest stability (smallest fold change between the groups). This miRNA, hsa-miR-30a-5p, turned out to be remarkably stable compared to U6 and RNU48 and was hence used for normalization. We believe that this approach could be developed for individual diseases. Nowadays, the majority of the studies use as normalization controls either those miRNAs that are detected relatively stable in all samples or exogenous controls (eg the spike-in of a known exogenous miRNA sequence such as cel-miR-39 from Caenorhabditis elegans for human samples). The drawback of the use of exogenous controls is that dilution of miRNAs (especially urine in for example in diabetic nephropathy) is not normalized. A combination of spiked- in to determine extraction efficiency and internal miRNA normalization might currently be the best choice.

Although urine clearly contains miRNAs, they are extremely diluted, hence difficult to detect. These difficulties in urinary miRNA analyses are exacerbated by the methods used for the extraction of miRNAs which yield total RNA where miRNAs represent only 0.1% of the total RNA extracted making it extremely difficult to calculate the amount of miRNA in the extract to be equivalent in all samples. Hence normalization is critical in determining urinary miRNAs. Even with the developed endogenous normalization procedure, within the same group of patients analyzed with the same technology, we observe a large variation in urinary abundance of miRNAs. This pending issue with normalization and variability most likely hampers quick evolution of the use of urinary miRNAs as biomarkers of (kidney) disease.

The use of different technologies for discovery and validation might be another cause of the low number of miRNAs validated in the CKD progression study. Next Generation Sequencing (NGS) uses a standardized method for normalization based on global signal normalization (less variability) and is currently significantly more sensitive than Taqman Low Density Arrays (TLDA) technology. For this reason it is possible that we were not able to validate a number of candidates using the TLDA approach. Increased patient numbers and technologies mimicking single cell PCR analysis might solve these issues.

7.2 miRNAs in kidney disease

Chronic kidney disease (CKD) is a complex pathology, especially in our study where we included CKD derived from different etiologies, with still unknown mechanisms leading to progression of disease. We tried in this study to determine the role of miRNAs in CKD progression and possibly provide novel biomarkers of progressive disease and therapeutic targets. In our retrospective study, urine samples from patients with progressive CKD vs non progressive CKD were used to investigate miRNAs in relation to CKD. The miRNA that was significant in both the discovery and validation study using two different technologies was miR-145-5p. Using *in vitro* knockdown studies the most likely mechanism linking mir-145-5p to CKD progression is necrosis.

The ureteropelvic junction (UPJ) obstruction project proposed a novel study plan to examine pathologies with difficulty to collect samples. UPJ is mainly a condition affecting newborn children and still it is not clear when the condition of the obstruction is severe and needs a surgery or can be dealt with caution. It is logical that obtaining kidney samples from newborn children, healthy and with UPJ, is not easy. Thus, taking into advantage the conservation ability of the miRNAs between the species we could combine the miRNA results from human urine samples (control and with UPJ obstruction) with mice tissue samples of the neonatal partial

unilateral ureteral obstruction (UUO) model. Our data revealed let-7a and miR-29b as molecules potentially involved in the development of fibrosis in UPJ obstruction via the control of DTX4 in both man and mice that would not be identified otherwise. The logical question in the design of this project is "how someone can be sure that the miRNAs in mice kidney are the same with the human in urine?". This is a question which could be answered only if we had available human tissue samples, but the bibliography on the 2 strongest candidates, (let-7a-5p and miR-29b-3p) is in favour of their presence in human kidney tissue as well in the pathological condition.

In both projects we faced the aforementioned drawbacks of normalization and putative miRNA quantity in the extracted RNA. To deal with the quantity of the miRNAs in the extracted total RNA, exosomal miRNAs could have been targeted, since exosomes can be isolated and include higher amount of "clean" miRNAs which can be measured with higher accuracy than in total RNA extract.

7.3 Conclusion and outlook

To conclude, urinary miRNAs are strong candidates to find use in diagnosis, prognosis, monitoring and therapy for kidney diseases. However, the majority of the available studies only show association between the urinary miRNA levels (increase or decrease) and renal disease clinical parameters without the verification of whether urinary miRNAs can be used to improve the diagnosis and/or prognosis. A correct workflow to promote the use of urinary miRNAs as biomarkers of kidney disease includes some well-defined steps taken from proteomics biomarker research: 1. Define the clinical question, 2. Choose carefully the sample set best associated to the clinical question, 3. Set up the experimental methodology, 4. Use appropriate statistics, 5. Confirm the findings in an independent test set (validation) and 6. Implement to clinical practice. To reach this goal, miRNA research needs to include larger cohorts compared to the previous studies (including ours) with clear definition of the pathology/etiology and prolonged disease follow-ups, expand/optimize the use of the technological tools available, establish a method for normalization and develop fast, reliable and reproducible protocols for the detection and validation of the miRNAs starting from smaller volumes of urine (less than 1 ml). A closer co-operation with bioinformatics is also suggested that will contribute significantly in identifying new miRNAs as well as definition of their potential targets, leading to optimized computation of the pathways controlled by specific miRNAs and ultimately to optimized selection of miRNAs for potential intervention.

miRNAs are here to stay and change the future on how we see and face pathologies and use them as biomarkers and as therapeutic targets now that many strategies against diseases,

including CKD, have reached a plateau. miRNAs have opened a new world in science and clinical practice. We just need to unlock their secrets.

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9. Annex

EXPERT REVIEWS

miRNAs in urine: a mirror image of kidney disease?

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miRNAs are short non-coding RNAs that control post-transcriptional regulation of gene expression. They are found ubiquitously in tissue and body fluids and participate in the pathogenesis of many diseases. Due to these characteristics and their stability, miRNAs could serve as biomarkers of different pathologies of the kidney. Urine is a non-invasive reservoir of molecules, especially indicative of the urinary system. In this review, we focus on urinary miRNAs and their potential to serve as biomarkers in kidney disease. Past studies show that urinary miRNAs correlate with renal dysfunctions and with processes involved in the pathophysiology. However, these studies also stress the need for future research focusing on large-scale studies to confirm the usability of urinary miRNAs as diagnostic and/or prognostic markers of different kidney diseases in clinical practice.

KEYWORDS: biomarker • canonical pathways • computational analysis • kidney • kidney disease • miRNA • urine

The year 1993 will remain in history as the beginning of a new research field in biological sciences. In that year, the teams of Ambros and Ruvkun independently published their discoveries on lin-4 [1–3]. The lin-4 gene produces a small transcript that does not encode a protein but negatively regulates the level of the LIN-14 protein involved in the development of *Caenorhabditis elegans* [1–3]. Since these first publications, 7 years passed until a second small non-coding RNA, let-7, was discovered with a similar function to lin-4 [4]. Since then, the now known microRNAs or miRNAs have stimulated a growing scientific interest and research has evolved significantly towards detecting and identifying these small molecules since miRNAs have been shown to be involved in a variety of physiological functions and diseases [5].

miRNA biogenesis, function & annotation

miRNAs are short (20–23 nucleotides in length), non-coding, endogenous, single-stranded RNA molecules. The biogenesis and the function of miRNAs have been extensively reviewed elsewhere [6,7]. In brief, miRNAs inhibit protein synthesis of protein-coding genes, either by inhibition of translation (if there is little complementarity with 3′-untranslated region of the mRNA) or by mRNA degradation (if there is extensive complementarity with the mRNA target) [5,7,8]. miRNA precursors (pre-miRNA) are transcribed from their specific non-coding gene or from introns of coding genes in the form of a cistronic or polycistronic transcript (pri-miRNA). After a first cleavage by the Drosha–DiGeorge syndrome critical region 8 complex, the pre-miRNA is produced and adopts a characteristic hairpin loop structure (FIGURE 1). Alternatively, the pre-miRNA is generated by splicing the host mRNA transcript. This non-canonical biogenesis pathway bypasses the cleavage by Drosha. In brief, the transcript is spliced in a complex called spliceosome, producing the 'mirtron' and next, the mirtron refolds to the typical pre-miRNA hairpin form [9].

In the next step, the pre-miRNA is transferred through exportin-5 from the nucleus to the cytoplasm. Subsequently, the DICER complex binds and cleaves the double-stranded pre-miRNA, and releases the mature single-stranded miRNA. From this point, the mature miRNA is ready to perform its biological function. Together with other proteins (the most important of them is Ago2), it forms the RNA-induced silencing complex (RISC), which guides the miRNA to the mRNA target, helps to connect to the 3′-untranslated region and blocks the translation of the mRNA [6–8,10]. Interestingly, one miRNA gene can lead to the generation of more than one

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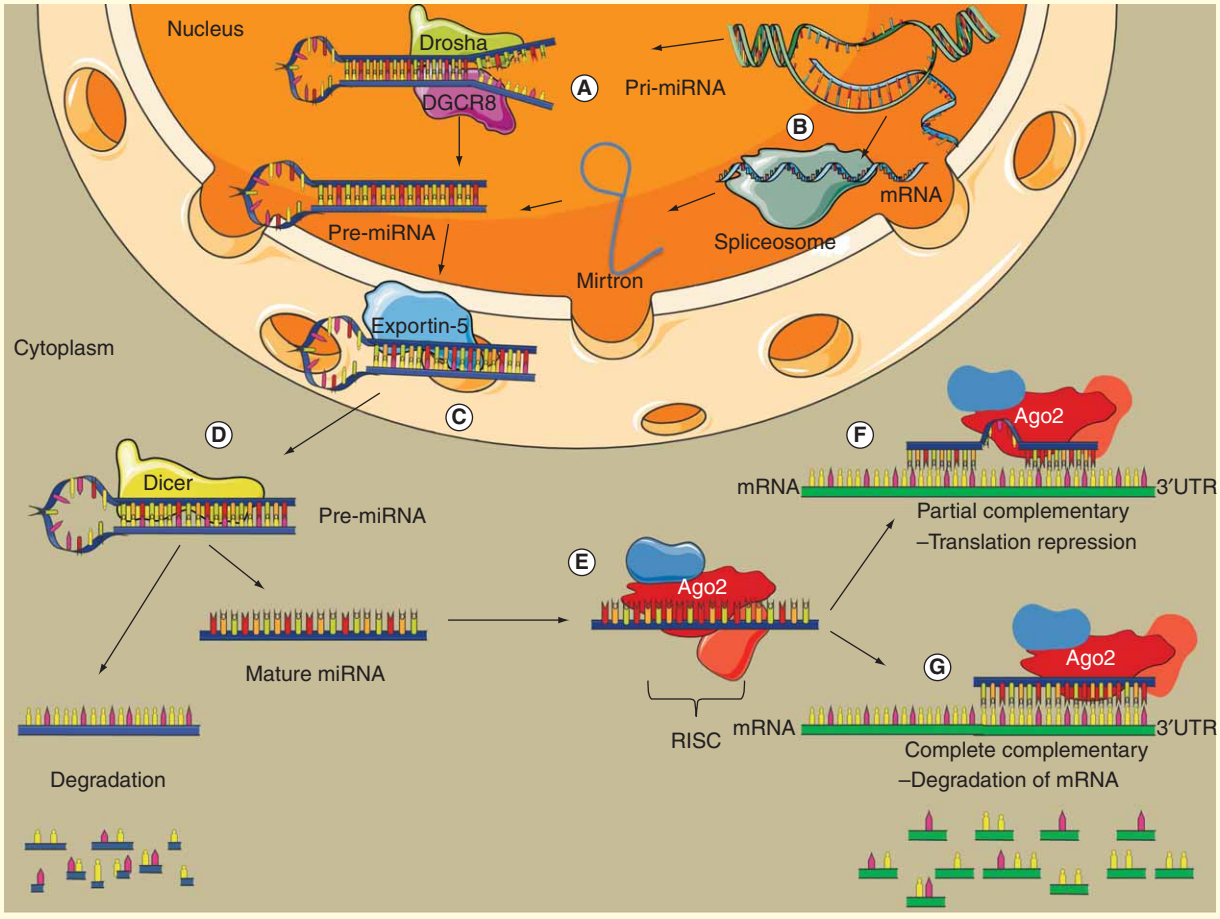

Figure 1. Biogenesis and function of the miRNA. (A) Canonical pathway: In the first step of miRNA biogenesis, miRNA is transcribed in a primary form (pri-miRNA with a hairpin loop). The Drosha–DGCR8 complex splices the pri-miRNA to form the precursor miRNA (pre-miRNA). **(B)** Non-canonical pathway: the miRNA is spliced from mRNA by a complex called spliceosome creating the mirtron. Next, the mirtron reforms to the shape of pre-miRNA, avoiding cleavage by Drosha. **(C)** The pre-miRNA is transferred from the nucleus to the cytoplasm with the help of Exportin-5. **(D)** In the cytoplasm the protein DICER cleaves the hairpin loop and releases the two strands. One strand will serve as the mature miRNA (18–22 nt length), while the other will be degraded. **(E)** The mature miRNA then binds to a protein complex including Ago2, forming the so-called RISC complex. Ago2 then connects the miRNA with the mRNA target: **(F)** if the miRNA sequence is partially complementary to the mRNA's 3′-UTR, it binds to it and represses translation. **(G)** If the miRNA sequence is completely complementary to the 3′-UTR of the mRNA, it binds and Ago2 cleaves the mRNA, stopping the translation of the protein. The picture was designed using Servier Medical Art.
3′-UTR: 3′-untranslated region; Ago2: Argonaute 2; DGCR8: DiGeorge syndrome critical region 8; RISC: RNA-induced silencing complex.

mature miRNA, and on the other hand, different miRNA genes can be clustered into the so-called miRNA families based on the miRNA sequence [11].

The continuously increasing number of reports on miRNAs, starting from some hundreds per year until 2007 and reaching approximately 7000 in 2013 and 2014, points to their potential role in the control of gene expression. This increase can be justified by the use of next-generation sequencing technologies combined with easy-to-use methods for extraction, quantification and expression of miRNAs. The number of newly identified miRNAs has been also rising at a fast pace, as shown by the number of entries in miRBase, which in the last update of June 2014 (version 21.0) reached 28,645 miRNA entries when considering all species, and 2588 entries for human miRNAs [12].

Advantages of urine miRNA measurement for kidney function

It has been shown that miRNAs are significantly more stable than mRNA, suggesting the potential suitability of miRNAs as biomarkers [13]. Among the different body fluids, miRNAs are present in urine, making the urinary miRNA pool potentially suitable to monitor kidney function and detect both renal and non-renal diseases [14,15]. Kidney diseases are a major and constantly rising worldwide health problem. The main obstacle in treating kidney diseases is the absence of early clinical signs or biomarkers before the kidney enters an irreversible dysfunction [16]. Nowadays, renal biopsy is the gold standard diagnostic tool but this is an invasive procedure that is associated with significant hemorrhagic risk [16]. Alternatively, urine can be

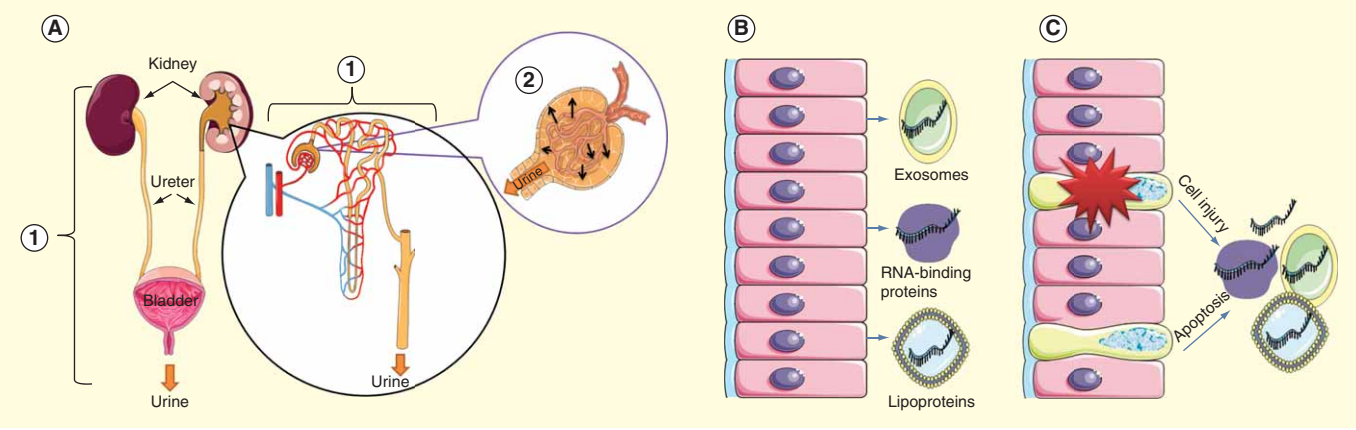

Figure 2. Possible origin of urinary miRNAs and miRNA transporting molecules. (A) It has been hypothesized that miRNAs are likely both shed from along the urinary pathway (1) and filtered from plasma (2). **(B)** miRNAs are produced by exocytosis and thus secreted in urine are most likely protected from degradation by existing in exosomes or lipoproteins (e.g., HDL) or bound with RNA-binding proteins (e.g., Ago2). **(C)** miRNAs (free or in/or attached to the RNA-binding proteins) can also be released in urine after cell injury or apoptosis. The figures were designed using Servier Medical Art.

collected easily, non-invasively and with low cost [15]. In addition, miRNAs in urine have been shown to be relatively stable under different storage conditions and resistant to repeated freeze–thaw cycles [17–19].

Although the origin of the urinary miRNAs has not been thoroughly studied, it has been hypothesized that they are likely both shed from along the urinary pathway and filtered from plasma (Figure 2A) [20–22]. miRNAs have been found to be secreted from the cells in which they are produced and packed in microparticles, such as microvesicles, exosomes and apoptotic bodies, and also bound to RNA-binding proteins or lipoprotein complexes (Figure 2B) [23]. Among those, exosomes are the best-studied miRNA vehicles. Exosomes are multi-vesicular vacuoles that transfer cellular components between cells and promote cell-to-cell communication and interactions [24,25]. Exosomes are also the 'bodyguards' of miRNAs by protecting them from the RNAse activity and the unfriendly urine environment. Cheng *et al.* reported that miRNAs enriched in exosomes from urine are protected from degradation and are capable of transferring the genetic information more efficiently than cell-free miRNAs, which are probably degraded by RNAse [15,26]. The protective role of other miRNA binding proteins has been studied in less detail but these few reports support the theory of protective transfer by these binding proteins of miRNAs in body fluids and in cell-to-cell communication. In particular, RNA-binding protein Ago2, in addition to being part of the RISC silencing complex (Figure 1), has been described to be involved in miRNA transport in the circulation, potentially leading to the transport of a functional miRNA-induced silencing complex [27]. In the same category of RNA-binding proteins, nucleophosmin 1 was also found to carry miRNA in serum in humans and possibly serve in cell-to-cell communication [28]. Finally, Vickers *et al.* revealed the surprising role of HDL lipoproteins in the transport of miRNAs in a similar way to these RNA-binding proteins [29]. Interestingly, apoptotic cells seem to signal using miRNAs. Upon apoptosis and cell injury, it has been shown that miRNAs bound to these previously mentioned RNA-binding proteins are released in the circulation (Figure 2C) [21,30].

Extraction of urinary miRNAs is quite straightforward and the majority of the past studies follow protocols based on the same principle as for the extraction of urinary mRNAs. Initially, protocols used tens of milliliters of urine, but currently this can be as little as 200 l using commercially available kits. miRNAs can be extracted from total urine, including the urinary cells, exosomes and miRNAs bound to RNA-binding proteins. This is the most used method (Supplementary Table 1 [Supplementary material can be found online at www.informahealthcare.com/10.1586/14737159.2015.1009449_Suppl.pdf]). The currently available commercial kits provide rapid extraction of total miRNAs from urine based on binding of small RNAs to specific material packed in columns. Other studies have exclusively focused on the urinary exosome content. The two classical ways to isolate exosomes were by cumbersome sucrose gradient ultracentrifugation or by isolation using antibodies specific for one of the surface proteins typically found on exosomes. The last method is very specific but might exclude some subfractions of exosomes. Currently, specific commercial kits, based on precipitation, allow easy and rapid exosomal miRNA isolation. A drawback of these kits compared to ultracentrifugation at ~200,000 g is that any precipitated material will be included in the 'exosomal' fraction and thus potentially less pure than the exosomal fraction obtained by ultracentrifugation. For the detection of isolated miRNAs, the most popular method is (semi)quantitative RT-PCR. For screening purposes, microarrays were popular in the past but are being slowly replaced by next-generation sequencing that allows the identification of truly novel miRNAs and has in general a higher sensitivity than the microarrays.

Altogether, these observations strongly suggest that urinary miRNAs could be a promising pool of non-invasive biomarkers, especially for diseases of the kidney and the urinary

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tract and thus mirror kidney (dys)function. Research in urinary miRNAs produces continuously new identifications and correlations of miRNAs with clinical features of kidney diseases.

Modification of urinary miRNA abundance in kidney diseases

Alterations in the expression of miRNAs affect kidney development and function [31–34]. This was exemplified by the report by Nagalakshmi *et al.* where knocking down DICER in mice caused nephron maldevelopment [35]. Also, the abundance of human kidney biopsy miRNAs has been reported as being modified in different kidney diseases [36–39]. In contrast, reports on human urinary miRNAs related to kidney diseases are limited (Supplementary Table 1). We discuss below the major discoveries recently made in this domain.

Chronic kidney disease

Chronic kidney disease (CKD) is a general term for progressive kidney failure from different causes and is classified into five stages defined by the decline in the glomerular filtration rate [40]. The final stage of CKD progression is called end-stage renal disease (ESRD), where the kidney stops functioning, a condition with increasing incidence worldwide [41]. At this stage, hemodialysis and transplantation are the only replacement therapies. A main issue in CKD is the early detection of disease in patients at risk (i.e., in individuals with diabetes, hypertension, etc.) and identify those patients with already established CKD that rapidly progress to ESRD [42,43].

A number of studies reported association of significant variations in urinary miRNA abundance in CKD. One of the first reports on urinary miRNAs in CKD described significantly increased levels of miR-638 in urine samples from patients with stage 3, 4 and 5 (ESRD) compared to samples from healthy volunteers [44]. MiR-638 has been shown to be associated with the maintenance of the epithelial cell phenotype [45]. In another report, differential urinary miRNA abundance was found to be associated with different CKD etiologies: reduced levels of urinary miR-15 were observed in diabetic glomerulosclerosis, increased levels of miR-17 in IgA nephropathy (IgAN) and increased miR-216a and miR-21 levels in hypertensive nephrosclerosis [46]. More importantly, urinary miR-21 and miR-216a abundance was correlated with the rate of renal function decline [46]. Among these miRNAs, a decrease in plasma miR-15b has been reported to be associated with hyperphosphatemia in patients with ESRD [47] and miR-21 is known to participate in the development of kidney fibrosis, a key mechanism leading to CKD [48–51].

Finally, reduced urinary abundance of miR-29a, miR-29b, miR-29c and miR-200a, miR-200b and miR-200c has been observed in CKD patients originating from a variety of etiologies, including diabetic nephropathy (DN), focal segmental glomerulosclerosis (FSGS), IgAN, membranous nephropathy and mesangial proliferative glomerulonephritis [52–56]. As miR-21, these miRNAs are known to be associated with the development of kidney fibrosis. miR-29 is reported to control the expression of tropomyosin 1, a protein involved in the epithelial to mesenchymal transition (EMT) mechanism, and collagen isoform COL2A1 (collagen is a well-documented protein that causes fibrosis) [57,58]. The mir-8 family (miR-200a, miR-200c, miR-141, miR-429) is found to target ZEB1, ZEB2 and SIP1 known to be involved in the EMT process, fibrosis and maintenance of the cell phenotype [59,60].

Diabetic nephropathy

About 25–40% of patients suffering from diabetes, either Type 1 (T1D) or Type 2 (T2D), will be diagnosed later with DN [61]. The pathophysiology of DN involves progressive thickening of the glomerular basement membrane, podocyte loss and mesangial proliferation in the glomerulus, and tubulointerstitial fibrosis characterized by uncontrolled deposition of extracellular matrix and low-grade inflammation [61–63].

Argyropoulos *et al.* studied urinary miRNA abundance in either normo-, micro- or macroalbuminuric T1D patients and in patients with overt DN. They observed that urinary levels of miR-323b-5p, miR-429 and miR-17-5p were associated with persistent microalbuminuria in patients with long-standing T1D [64]. After predicting the targets of these miRNAs, it was hypothesized that miR-323b-5p may regulate Claudin-16, which is a vital component of the tight junction in the thick ascending limb of Henle in the tubular section of the kidney [65] and that miR-17-5p targets fibronectin [66], an extracellular matrix protein increased in DN [67]. In addition, miR-429 belongs to the same fibrosis-related mir-8 family mentioned above [68].

Another study analyzed the abundance of urinary miRNAs in urine of T1D patients with microalbuminuria as well [69]. The authors observed decreased urinary levels of exosomal miR-155 and miR-424 and increased levels of miR-145 in T1D patients with microalbuminuria compared to T1D patients with normoalbuminuria or non-diabetic controls [69]. Interestingly, *in vivo* and *in vitro* studies suggest that glomerular miR-145 overexpression might be triggered by hyperglycemia, potentially stimulating mesangial cell hypertrophy and cytoskeleton remodeling, key early features of DN [70,71]. miR-424 was found to be involved in the regulation of angiogenesis [72,73] and miR-155 has been shown to regulate angiotensin 2 [74] and SMAD proteins related to fibrosis [75,76].

Besides glomerular and interstitial damage, DN is also characterized by vascular lesions. Liu *et al.* found that miR-126 abundance was increased in urine of T2D patients suffering from DN compared to diabetic patients without DN and healthy volunteers. miR-126 is a miRNA highly enriched in endothelial cells and is a key player in maintaining endothelial homeostasis and vascular integrity. The authors, therefore, suggested that urinary miR-126 originates from endothelial cells-derived exosomes and propose that urinary miR-126 could serve as a biomarker to identify T2D patients with DN and monitor progression of the disease [77], although this remains to be confirmed in independent studies.

Diabetes is the number one cause of DN. The reports on the connection of diabetes and DN with urinary miRNAs are

promising, but further investigation is needed to determine if urinary miRNAs can be used as biomarkers to predict the initiation of DN because of diabetes.

Glomerulopathies

Primary glomerulopathies, such as minimal change disease, FSGS, membranous glomerulonephritis, IgAN and membranoproliferative glomerulonephritis, are disorders affecting the glomerular structure and function and are considered as a leading cause of ESRD and major cause of CKD worldwide [78,79].

IgA nephropathy (Berger's disease)

IgAN is the most frequent glomerular disease and is characterized by mesangioproliferative changes in glomeruli with deposition of immunoglobulin A in the mesangium [80].

Research on miRNAs in urine of IgAN patients detected decreased abundance of miR-200a, miR-200b and miR-429 (mir-8 family) and correlated with disease severity and rate of progression. Decreased urinary levels of miR-429 were also found to be correlated with an increased rate of renal function decline [81]. Furthermore, it has been shown that increased levels of miR-146a and miR-155 in urine and tissue are associated with reduced production of pro-inflammatory cytokines, IL-1β, IL-6 and tumor necrosis factor (TNF-α), molecules involved in the pathogenesis of IgAN [82–84]. Finally, urinary levels of miR-21, miR-29b, miR-29c and miR-93, mostly fibrosis-related miRNAs, correlated with urinary SMAD3 (but not with TGF-1, SMAD2, SMAD4 or SMAD7) in IgAN patients [52]. This observation supports the involvement of the TGF-β1/SMAD mechanism in the progression of renal fibrosis during IgAN [57].

Other glomerulopathies

Wang *et al.* demonstrated that the levels of urinary miR-10a and miR-30d were elevated in patients suffering from FSGS compared to healthy donors [85]. miR-10a and miR-30d are reported as kidney-specific miRNAs serving a protective role against kidney tissue injuries by targeting IL-12/IL-23p40 [86], the pro-apoptotic protein Bim [87], apoptotic caspase CASP3 [88] and tumor suppressor gene p53 [89].

In another report, urinary miR-155, miR-196a, miR-30a-5p and miR-490 levels have been shown to increase in FSGS patients compared to healthy controls, and moreover miR-196a, miR-30a-5p and miR-490 levels significantly decreased in FSGS patients under treatment with steroids, a common immunosuppressive therapy employed in FSGS patients leading to remission [90], suggesting a potentially predictive use for the response on steroid therapy [91].

Renal allograft monitoring

Up to now, the most effective renal therapy for ESRD is renal transplantation [92]. However, even though renal transplantation is a very successful short-term therapy, up to 50% of the grafts lose function after 10 years. The most common features reported in rejection of grafts are interstitial fibrosis, tubular atrophy and kidney dysfunction, appearing after the first year of transplantation [39,93].

Urinary miRNA miR-210 is a possible candidate to monitor acute allograft rejection since its urinary abundance decreased after rejection compared to stable transplant [94]. miR-210 is involved in the endothelial cell response to hypoxia [95] and was found to be an indicator of cellular damage in acute kidney injury (AKI) [96].

Urinary abundance of miR-200b, miR-125b, miR-203, miR-211, mir-30 family and miR-20 is decreased, while the abundance of miR-142-3p is increased in chronic kidney allograft rejection [97,98] The functions of these miRNAs have been related to pathological processes in chronic kidney allograft rejection. miR-200b, as mentioned previously, is related to fibrosis, a mechanism present in allograft rejection and miR-125b is involved in B-lymphocyte development [99], in macrophage activation [100] and involved in the maintenance of the smooth muscle phenotype of the juxtaglomerular cells [101]. miR-203 was shown to regulate the innate immune response [102] and miR-142 is proposed to be a key element of the regulation of T cells and macrophages [103,104]. Finally, miR-204 is reported to be involved in apoptosis and cell growth [105] and the mir-30 family was proposed to be involved in the maintenance of podocytes and glomerular function [106].

Renal allograft monitoring is still a major issue in renal transplantation and these studies suggest that specific urinary miRNAs are associated with rejection. It, however, remains to be determined whether these urinary miRNAs can be predictive markers.

Acute kidney injury

AKI represents a sudden dysfunction of the kidney leading to disruption of electrolyte, fluid and metabolic homeostasis for a few hours and up to a few weeks [107]. Risk factors for AKI include non-modifiable factors like age (more frequent in the elderly), pre-existing proteinuria and CKD, co-morbid diseases and modifiable factors such as exposure to nephrotoxins, contrast media, sepsis and major surgery [107].

Three studies examined modified urinary miRNA abundance in AKI. Among the miRNAs detected at significantly different levels compared to healthy individuals, miR-155 (decreased) and miR-21 (increased) stand out in relation to the development of AKI [108,109]. miR-21 has been shown to be involved in many pathological conditions, including inflammation, proliferation, fibrosis and apoptosis [110–112], while miR-155 is involved in the regulation of the inflammatory response [108]. Finally, the elevated level of miR-494 after ischemia/reperfusion injury was correlated with inflammation by inhibiting its direct target, activating transcription factor 3 [113].

Lupus nephritis

Lupus nephritis (LN) is one of the most severe manifestations of systemic lupus erythematosus, an autoimmune disease with the ability to affect any organ or tissue. The pathogenesis of LN is a combination of various pathological processes resulting

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Table 1. The top canonical pathways as generated from the miRNA data set lists for each disease by ingenuity pathway analysis.

Canonical pathways	Sum of diseases	ADPKD	AKI	CKD	DN	FSGS_MCD_MGN	IgAN	LN	RAR
Role of macrophages, fibroblasts and endothelial cells	3		1				1	1	
Regulation of the epithelial–mesenchymal transition pathway	3		1			1			1
PI3K/AKT signaling	3	1			1	1			
Cell cycle G1/S checkpoint regulation	3			1	1				1
IL-6 signaling	2						1	1	
Dendritic cell maturation	2						1	1	
Fibrosis	2			1			1		
Aryl hydrocarbon receptor signaling	1				1				
Integrin-linked kinase signaling	1	1							
Toll-like receptor signaling	1							1	
PTEN signaling	1	1							
Tec kinase signaling	1					1			
IL-10 signaling	1						1		

Pathways with a clear reference to cancer were omitted.
ADPKD: Autosomal dominant polycystic kidney disease; AKI: Acute kidney injury; CKD: Chronic kidney disease; DN: Diabetic nephropathy; FSGS: Focal segmental glomerulosclerosis; IgAN: IgA nephropathy; LN: Lupus nephritis; MCD: Minimal change disease; MGN: Membranous glomerulonephritis; PTEN: Phosphate and tensin homolog; RAR: Renal allograft rejection.

in tubular damage, tubulointerstitial inflammation and fibrosis. All processes involve modification of miRNA expression [114] and consequently miRNAs have been scrutinized for their involvement in the development of LN [115], mostly in blood cells and renal biopsies [116–118]. However, only a few reports are available on LN and urinary miRNA.

The available publications are referring to experiments using urine samples from patients with LN and healthy volunteers.

Table 2. Top nine of most frequently found urinary miRNAs in studies related to kidney disease (>3 studies).

miRNA	Mentioned in number of studies
hsa-mir-8	7
hsa-mir-155	5
hsa-mir-30	4
hsa-mir-638	4
hsa-mir-10	3
hsa-mir-146	4
hsa-mir-29	3
hsa-mir-21	3
hsa-mir-423	3

The miRNAs are presented as families. Total number of studies reviewed, n = 22.

miR-146a and miR-155 are proposed as potential markers for diagnosis, disease activity and therapeutic response for LN [119,120]. miR-146a and miR-155 have been extensively studied and their role in innate immune response has been shown repeatedly [111,121,122]. miR-146a has been reported to regulate the Type I interferon pathway, a pathway involved in LN pathogenesis [123] and miR-155 is a regulator of T and B cell activation [83,124], cells with a key role in development and progression of LN [125].

Polycystic kidney disease

Polycystic kidney disease (PKD) either autosomal dominant (ADPKD) or autosomal recessive is characterized by the formation of multiple benign cysts causing the kidney to enlarge and to progressively loose structure and function [126]. Fifty percent of patients diagnosed with ADPKD will progress to ESRD around the fifth decade of their life [127,128].

To our knowledge, only one study has investigated the abundance of miRNA in urine of ADPKD patients. Urinary miR-1 and miR-133a were found with lower abundance in ADPKD patients compared to patients with CKD originating from other etiologies [129]. These miRNAs have been identified in renal cell carcinoma as tumor suppressors controlling cell proliferation and apoptosis [130]. Also, the significantly increased urinary levels of miR-223, miR-199a and miR-199b were found to be associated with monocyte infiltration and EMT, features of the pathogenesis of ADPKD [129,131–134].

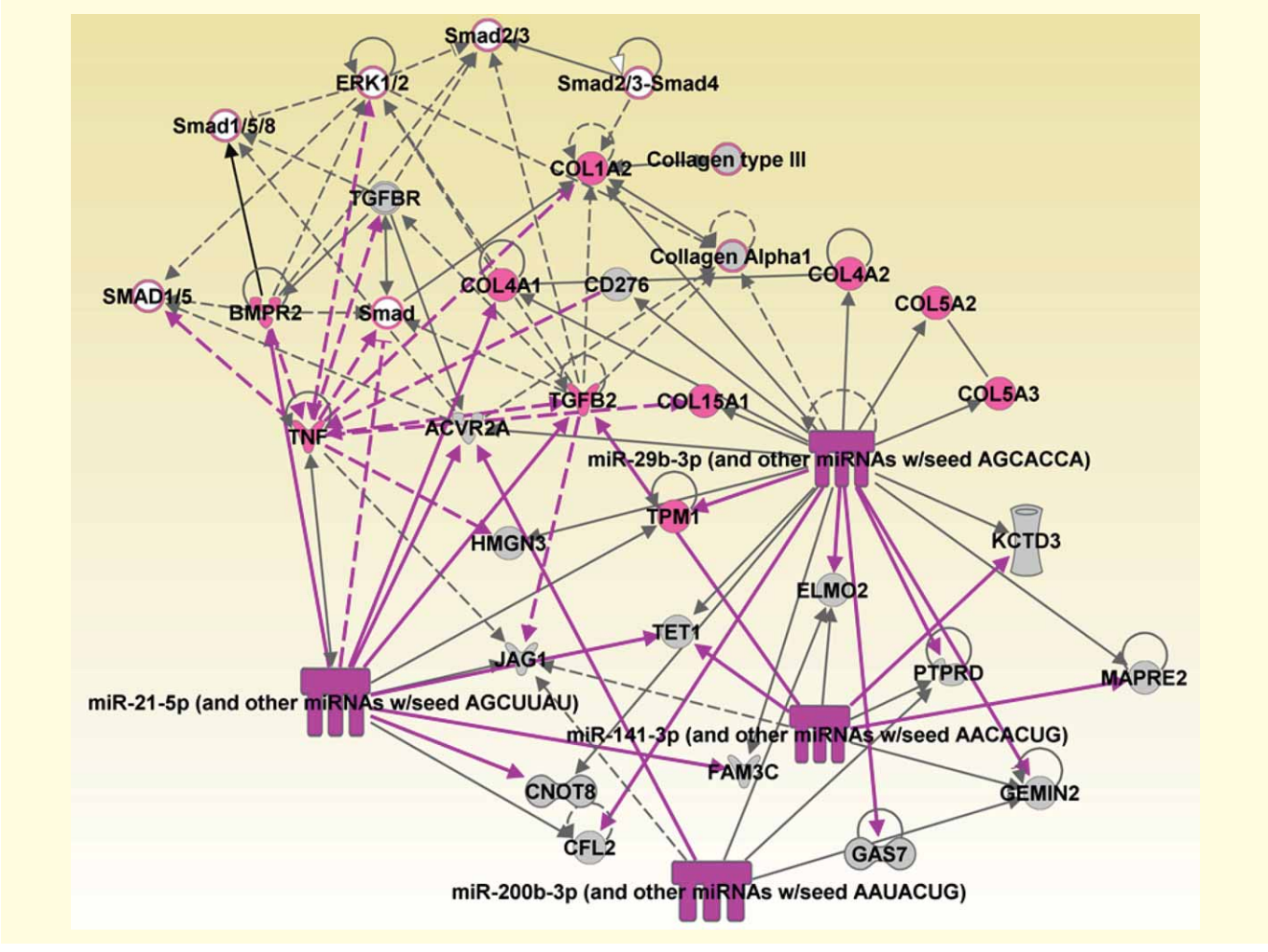

Figure 3. Network analysis of the most frequently observed urinary miRNAs associated with renal disease (Table 2, miR-21, mir-8 and miR-29 family). The purple arrows indicate the experimental observed direct targets of the miRNAs. Among the targets present are TGF-β and SMAD isoforms, collagen isoforms, ACVR2A, ERK1/2, TNF-α and BMPR2 reported to be involved in fibrotic mechanisms, a hallmark of renal disease. Figure generated with ingenuity pathway analysis.

Computational analysis

In an attempt to examine in depth the urinary miRNAs and their potential links to disease pathways in kidney disease, we performed a computational analysis through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, [135]) using all reported miRNAs with significantly changed urinary abundance in kidney disease (Supplementary Table 1). These miRNAs were uploaded in data sets according to the kidney disease in which they were detected, followed by miRNA target filtering analysis under strict filters (only experimentally observed miRNA–mRNA pairs were accepted and we excluded any association with cancer-related pathways). This produced information on the pathways most frequently observed and revealed that the targets of the miRNAs are involved in mechanisms that control major processes in kidney disease (Table 1).

Indeed, macrophages and fibroblasts have been observed in renal injury and fibrosis [136–138]. In addition, macrophages are known vehicles of miRNAs [139]. EMT is a well-known, although debated, mechanism involved in the development of renal fibrosis [140] and the PI3K/AKT signaling pathway was recently linked to the EMT process [141]. Finally, deregulation of the cell cycle also promotes fibrosis and renal injuries [142]. Given the distribution of these pathways over the different pathologies, these modified pathways do not appear to be associated with a specific kidney disease. This suggests that the miRNAs found in urine and their targets are involved in the general kidney (dys)function. Hence, the urinary miRNAs associated with these pathways might not be good biomarkers to distinguish between different renal diseases.

In contrast, some canonical pathways were only found enriched in specific kidney disease. For example, IL-6 and IL-10 signaling along with Toll-like receptor signaling were uniquely linked to IgAN and LN, diseases related to the immune response and inflammation, and miR-29, miR-146a and miR-155 are reported to regulate these pathways [143–147].

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Table 3. The top 13 family miRNAs that appear in most kidney diseases (complete list in Supplementary Table 3).

miRNA	No. of diseases observed	ADPKD	AKI	CKD	DN	FSGS/MCD	IgAN	LN	MGN	RAR
hsa-mir-8	7		✓	✓	✓	✓	✓	✓		✓
hsa-mir-638	5			✓	✓	✓			✓	✓
hsa-mir-155	4		✓			✓	✓	✓		
hsa-mir-17	4			✓	✓		✓			✓
hsa-let-7	3	✓	✓							✓
hsa-mir-154	3		✓		✓					✓
hsa-mir-1260	3				✓	✓				✓
hsa-mir-21	3		✓		✓	✓				
hsa-mir-29	3			✓	✓		✓			
hsa-mir-30	3				✓	✓				✓
hsa-mir-423	3		✓			✓				✓
hsa-mir-574	3				✓	✓	✓			
hsa-mir-92	3				✓	✓				✓

The members of the families are presented in Supplementary Table 4.
ADPKD: Autosomal dominant polycystic kidney disease; AKI: Acute kidney injury; CKD: Chronic kidney disease; DN: Diabetic nephropathy; FSGS: Focal segmental glomerulosclerosis; IgAN: IgA nephropathy; LN: Lupus nephritis; MCD: Minimal change disease; MGN: Membranous glomerulonephritis; RAR: Renal allograft rejection.

Another example is Tec kinase signaling, enriched in glomerulopathies. Tec kinase is shown to interact with SOCS-1 in glomerulopathies [148]. Finally, in ADPKD the integrin-linked kinase signaling and phosphate and tensin homolog (PTEN) signaling pathways were uniquely modified that appear to be involved in the pathogenesis of ADPKD. Integrin-linked kinase signaling has been reported to be involved in fibrogenesis. PTEN has been shown to enhance angiogenesis and supports proliferation, migration and adhesion [149,150] and is identified in a mechanism for Akt activation by TGF-β via PTEN down-regulation by miR-216a/217 [151].

Overall, this computational analysis supports the link of urinary miRNAs to processes ongoing *in situ* in kidney disease.

Expert commentary

The biological function of miRNAs, their involvement in regulation of gene expression, their ubiquitous presence and their stability have set miRNAs as the new 'hot trend' in biological research. According to Friedlander *et al.* nearly 2000 human miRNAs genes have been identified and it is more than likely that in the future this number will increase even more. In their very recent report they predicted more than 1000 additional miRNAs by using computational methods [152], suggesting that the potential pool of miRNAs as biomarkers of disease will be even larger in the near future and will expand their potential application.

The observations of involvement of miRNAs in the etiology of pathologies as diverse as cancer, neurodegenerative diseases, vascular aging and atherosclerosis are increasing [153–156]. The same tendency is observed for kidney diseases and urine might be a valuable source of miRNA-based biomarkers of kidney disease in addition to a source for other molecules of kidney disease including proteins, peptides and metabolites [157].

The overview of the recent literature and the computational analysis also show that the urinary miRNAs correlate overall well with knowledge on the ongoing kidney disease processes. The most frequently observed urinary miRNAs associated with renal disease currently are from the mir-8 and mir-29 family and miR-21 (observed in 7/22, 3/22 and 3/22 of the reviewed studies, Table 2). In an additional computational analysis, we focused on networks based on these miRNAs and their experimentally observed targets (Figure 3). The canonical pathway related to fibrosis had the lowest p-value (7.45×10^{-14}) and confirmed the relevance of these miRNAs in fibrosis, a hallmark in many kidney diseases. Furthermore, the network pointed to important validated fibrotic targets, which are directly connected to these miRNAs including TGF-β and SMAD isoforms, collagen isoforms, ACVR2A [158], ERK1/2 [159], TNF-α [160] and BMPR2 [161,162].

Additional urinary miRNAs that were frequently found with differential abundance in urine in a significant number of papers are miR-155 (5/22 studies), miR-638 (4/22 studies) and miR-30 (4/22 studies). Changes in expression of these miRNAs are also observed in kidney tissue during kidney development and kidney diseases, including DN, IgAN, LN and AKI [32] and linked to processes in these diseases as described above. The family members of mir-8 (miR-200 and miR-428) also appear associated with the majority of the kidney diseases (6/9 diseases)

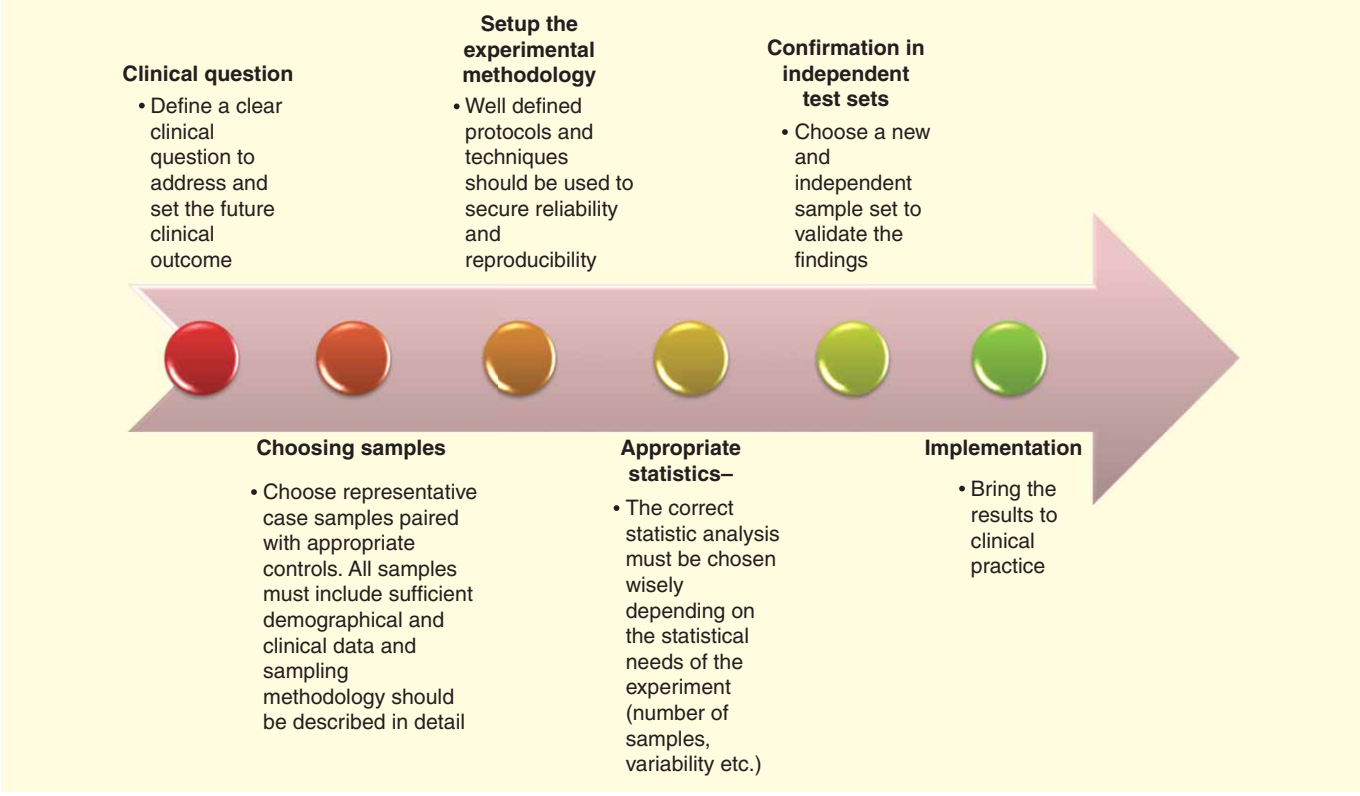

Figure 4. A possible workflow of how research on urinary miRNAs could lead to clinically useful urinary miRNAs.
Adapted from [164].
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enhancing the potential importance (e.g., known to be involved in AKI, DN, IgAN and LN) of this miRNA family in kidney physiology and disease (Table 3). The subsequently most frequently found miRNAs (in 5/9 diseases) are miR-638 (related to the function of the epithelial cell and found in DN and glomerulopathies) and miR-155 (known to interact with immune system cells and thus found mostly in IgAN and LN). These observations should be strengthened in the near future by the increasing knowledge of the targets of these different miRNAs. A complete list of the urinary miRNAs detected in different kidney diseases can be found in Supplementary Table 2.

It must be noted, however, that the direction in change of abundance of urinary miRNAs differs between renal diseases. For example, urinary miR-141 abundance was found to decrease in DN and increase in minimal change disease and LN. Conversely, miR-155 was observed with reduced abundance in urine of patients in DN and AKI but with increased abundance in IgAN and LN. Furthermore, it is interesting to point to the fact that although being modified in the largest number of different kidney diseases, the direction of change of the urinary abundance of miR-200 is variable among the kidney diseases studied. Similar observations hold for the key miRNAs, miR-29, miR-429 and miR-638. Although to date this behavior seems somewhat erratic, this observation is calling for further investigation but is in favor for the use of urinary miRNAs as potential markers of disease. To that direction, Konta *et al.* presented in their study that it is possible to record a pattern of urinary miRNAs in different kidney diseases [163] showing the potential of these molecules for diagnostic purposes.

Five-year view

The apparent ability of miRNAs to mirror biological function in body fluids during disease opens up a potential wide range of uses for urinary miRNAs in diagnosis, prognosis, monitoring and therapy for kidney diseases. However, most of the studies only show association between the urinary miRNA levels (increase or decrease) and renal disease clinical parameters without the verification of whether urinary miRNAs can be used to improve the diagnosis and/or prognosis. In order to improve upon urinary miRNA studies and promote the use of urinary miRNAs as biomarkers of kidney disease, miRNA research could 'borrow' a well-established workflow from clinical proteomics biomarker research. This workflow includes specific and well-defined steps: Define the clinical question, choose carefully the sample set best associated with the clinical question, set up the experimental methodology, use appropriate statistics, confirm the findings in an independent test set (validation) and implement to clinical practice (Figure 4) [164]. To reach this goal, research in the next years should focus on studies involving larger cohorts compared to the previous studies with clear definition of the pathology/etiology and prolonged disease follow-up. Furthermore, we need to expand/optimize the use of the

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technological tools available (RT-PCR is used in the majority of the available studies).

Finally, it appears that bioinformatics will contribute significantly to the field in the coming years both in identifying new miRNAs as well as definition of their potential targets, leading to optimized computation of the pathways controlled by specific miRNAs and ultimately to optimized selection of miRNAs for potential intervention.

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Key issues

- miRNAs are short (20–23 nucleotides length), non-coding, endogenous, single-stranded RNA molecules that inhibit protein synthesis of protein-coding genes.
- miRNAs are produced in all cells and can be found in almost all body fluids, either free or bound to proteins, microvesicles, exosomes and apoptotic bodies.
- The involvement of miRNAs in a large variety of biological functions combined with their great stability potentially makes them suitable molecules for diagnosis, prognosis and monitoring of disease.
- Urine is a biofluid which is collected non-invasively and might be a valuable source of miRNA-based biomarkers of kidney disease in addition to a source for other molecules of kidney disease, including proteins, peptides and metabolites.
- Although the reports on urinary miRNA and kidney disease are limited, the available information both in the literature and obtained using a specific computational pathway analysis carried out for the review suggest that urinary miRNAs reflect on-going known disease processes in the kidney and hence can potentially serve as non-invasive biomarkers of kidney disease.
- The majority of the urinary miRNAs were linked to one of the hallmarks of kidney disease which is fibrosis.
- Future research should focus on large-scale studies to confirm the usability of urinary miRNAs as diagnostic and/or prognostic markers of different kidney diseases.

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