Western Norway University of Applied Sciences

BACHELOR'S THESIS

Differential expression of microRNAs correlated to diabetic kidney disease.

A literature review.

Allette Steffensen Alværvik

Stine Kamilla Ommundsen

Sondre Hellevang Berg

Institute of Security, Chemical Engineering and Biomedical Laboratory Science,
Faculty of Engineering and Natural Sciences
Supervisor: Gry Sjøholt
29.05.2020

We confirm that the work is self-prepared and that references/source references to all sources used in the work are provided, cf. Regulation relating to academic studies and examinations at the Western Norway University of Applied Sciences (HVL), § 12-1.

Abstract

Norwegian:

Diabetes Mellitus (DM) er en kronisk sykdom utbredt verden over, og er preget av utilstrekkelig insulinproduksjon eller ineffektiv insulinregulering av glukoseomsetning i blod. Pasienter med diabetes utvikler ofte nyresykdom, og behovet for en biomarkør som kan oppdage diabetisk nyresykdom (DKD) tidlig i forløpet har økt. I løpet av de siste årene har forskjellige microRNA (miRNA) blitt undersøkt som potensiell biomarkør. miRNA er små ikke-kodende RNA-molekyler som rapporteres å være involvert i regulering av alle biologiske prosesser ved å hemme proteinsyntese. De små RNA-molekylene kan ekstraheres fra celler, vev og forskjellige biologiske væsker. For å kvantifisere miRNA er den mest foretrukne metoden qPCR. Dette er en metode som er veletablert og ansett for å være både følsom og spesifikk. Kvantitativ PCR er en målrettet metode, som kvantifiserer forhåndsutvalgte miRNA-er, men metoden kan ikke identifisere nye miRNA-er slik som RNA-sekvensering. I denne litteraturstudien blir forskningsartikler som rapporterer differensielt uttrykk for miRNA i DKD gjennomgått. På grunn av variasjoner i forskningsdesign og spredte resultater fra flere forskningsgrupper, som alle markedsfører forskjellige kandidater, er det foreløpig ingen miRNA som har stått frem som en åpenbar biomarkør for fremtidig DKD-diagnostikk.

Stikkord: *microRNA, qPCR, diabetes mellitus, diabetisk nyresykdom (DKD), biomarkør*

English:

Diabetes mellitus (DM) is a worldwide spread chronic disease, characterized by insufficient insulin production or inefficient insulin regulation of glucose turn-over in blood. Patients with diabetes often develop kidney disease, and the need for a biomarker that can detect diabetic kidney disease (DKD) early in the course has increased. Over the past few years, various microRNAs (miRNAs) have been researched as potential biomarker. miRNAs are small non-coding RNA molecules that are reported to be involved in regulating all biological processes by inhibiting protein synthesis. The small RNA molecules can be extracted from cells, tissues and various biological fluids. To quantify miRNAs, the most preferred method is qPCR. This is a method that is well-established and considered to be both sensitive and specific. Quantitative PCR is a targeted method, quantifying pre-selected miRNAs and consequently cannot identify new miRNAs, which is different to the method of untargeted RNA-sequencing. In this literature study, research papers reporting differential expression of miRNAs in DKD are reviewed. Due to variations in research design and scattered results of several research groups, all marketing different candidates, so far there is no single miRNA that have stood out as an obvious biomarker of future DKD diagnostics.

Keywords: *microRNA, qPCR, diabetes mellitus, diabetic kidney disease (DKD), biomarker*

Preface

This bachelor thesis is written as part of a bachelor's degree in Biomedical Laboratory Science as part of the course BIO160, at the Faculty of Engineering and Natural Sciences at Western Norway University of Applied Sciences. This thesis was originally planned to be written in collaboration with Cape Peninsula University of Technology in South Africa, because of worldwide restrictions due to Covid-19 pandemic, the thesis was changed to a literature study. The choice of subject for the literature review is therefore inspired by our external supervisor in Cape Town, professor Tandi Matsha-Erasmus. Although the thesis was a little different than expected and more demanding due to lost time, we have had a good collaboration and learned a lot from the process.

We would like to give a big thank you to Gry Sjøholt that have been our internal supervisor through this project and guided us in the right direction. We would also like to thank Tandi Matsha-Erasmus, that was supposed to be our supervisor in South Africa, for giving us information and inspired us to learn more about microRNA.

Bergen, 29.05.20

Content

Introduction

MicroRNA

MicroRNAs (miRNAs) are small single-stranded RNAs with a length of 19 to 28 nucleotides (Cao et al., 2019). MicroRNAs regulate various cellular processes, through specific complementary binding to single stranded messenger-RNA (mRNA) and in this way preventing protein translation. In 1993 the first miRNA, *lin-4*, was discovered in the nematode *Caenorhabditis elegans* (*C.elegans*) and since, more than thousands of miRNA have been discovered in plant-, animals-, and viral genomes (Esquela-Kerscher, 2014). In 2002, the first study that could relate miRNA to a disease was published, showing that miR-15 and miR-16 are down-regulated in chronic lymphocytic leukemia (Calin et al., 2002).

In the cells, miRNAs are initially transcribed from DNA into primary-miRNAs (Pri-miRNAs) as illustrated in figure 1. The RNase III enzyme, Drosha and its binding partner, DiGeorge syndrome critical region gene 8 (DGCR8), binds to the Pri-miRNAs and process them into smaller molecules, precursor miRNAs (Pre-miRNAs) (Cao et al., 2019). Through Exportin 5, Pre-miRNAs are transferred from the nucleus into the cytoplasm and are processed by another RNase III enzyme, Dicer. Dicer collaborates with transactivating response RNA-binding protein (TRBP) to generate the mature miRNA duplex. Thereafter, one of the strands of the miRNA duplex binds to the RNA-induced silencing complex (RISC) (Cao et al., 2019). In this complex, single stranded miRNAs bind to complementary nucleotide sequence, often localized in the 3´-untranslated region of the target mRNA. Due to the specificity of this binding, this interaction induce translational repression (low specificity) or degradation (high specificity) of mRNA (Kato & Natarajan, 2014).

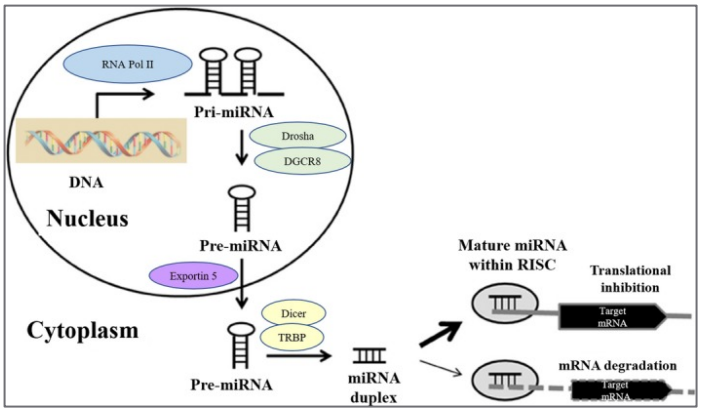

Figure 1: Biogenesis and repression of protein translation of miRNA (Cao et al., 2019).

In *Homo Sapiens* it is currently identified more than 1500 miRNA sequences (Badal & Danesh, 2014). It's been suggested that miRNAs may have hundreds of mRNA targets, this implying that multiple mRNAs may be repressed post-translationally or degraded by a single miRNA, and multiple miRNA can also bind to separate sites and cooperate on a single mRNA target (Badal & Danesh, 2014).

MicroRNA nomenclature

The first miRNA was identified in 1993, but it was not until 2001 that the term "microRNA" was introduced (Wright & Bruford, 2011). The letters that stands before the miRNA as shown in figure 2, represent the organism the miRNA is found in. In figure 2 "hsa" stands for *Homo sapiens* (Griffiths-Jones et al., 2006). Next is "miR", which is short for microRNA, and the following number "369" stands for the order of naming. The mature sequences of miRNA is labelled "miR", and the precursor hairpins, such as pre-miRNA and pri-miRNA, are labelled "mir" (Griffiths-Jones et al., 2006). Some miRNA sequences seem to be excised from opposite arms of the same hairpin precursor (Griffiths-Jones et al., 2006). These mature sequences are named by the arm, with 5p (5'arm) or like figure 2 shows 3p (3'arm). There are also some mature miRNA that are transcribed from different genes but have an identical sequence (Pritchard et al., 2012). These often have number suffix, for example hsa-miR-21-1, and hsa-miR-21-2. When you see a miRNA with a letter after it, like hsa-miR-20a vs hsa-miR-20b, it means that the mature miRNAs are closely related but differ by one or two nucleotides (Pritchard et al., 2012).

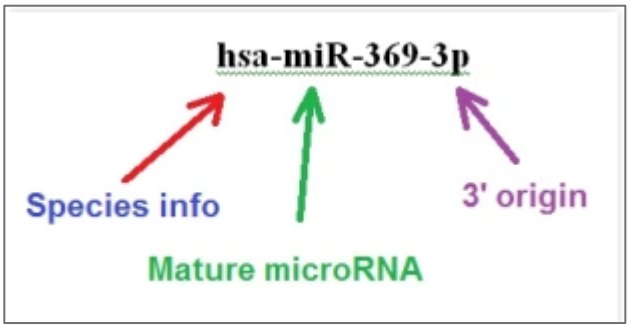

Figure 2: An example of the nomenclature for microRNA (Linse, 2013).

MicroRNAs are released from the cells as signalling molecules in exosomes

The cells in the body generates signaling molecules like miRNA and protein, that transduces essential information for other cells in the organism. The signaling molecules are then transmitted out of the cell by extracellular vesicles (EV) (J. Zhang et al., 2015). EV are released from the cell into body fluids such as plasma, serum, urine, and saliva. Exosomes are EVs with a diameter of 40-100 nm containing high concentrations of different signalling molecules. Exosomes are released into the extracellular compartment after fusion with the plasma membrane as figure 2 shows. Exosomes are initially formed by endocytosis (J. Zhang et al., 2015). The cell membrane is first internalized to produce endosomes, then many small vesicles are formed within the endosome by invading portions of the endosome membrane. These are called multivesicular bodies (MVB). The MVBs then fuse with the cell membrane and release the endosomal vesicles into the extracellular compartment to become exosomes (J. Zhang et al., 2015). Free exosomes will be engulfed by the cellular membrane, transporting different signaling molecules into the recipient cells (figure 3).

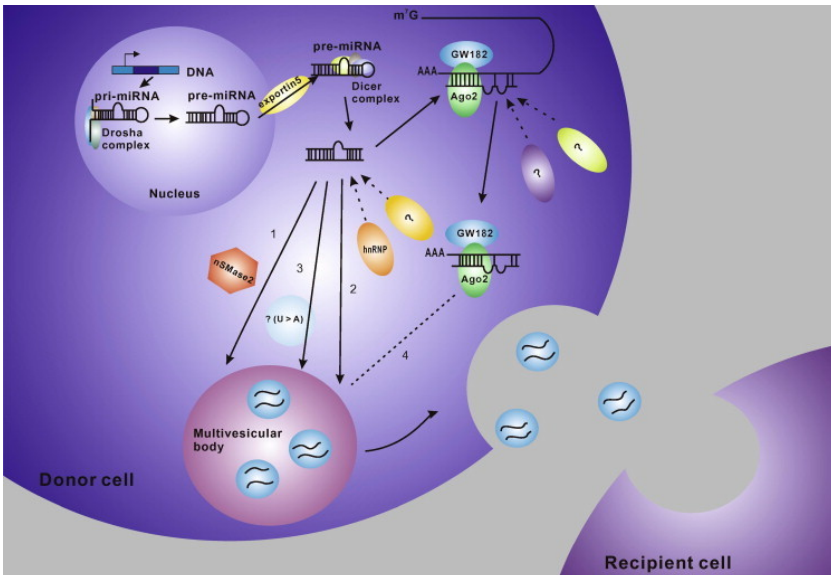

Figure 3: MicroRNA is formed in the cell and then transported into the extracellular compartment by exosome. The figure shows four different ways mature miRNA are sorted into exosomes : (1) nDMase2 dependent pathway; (2) sumoylated hnRNPs-dependent pathway; (3) 3´miRNA sequence-dependent pathway; (4) The miRISC-related pathway (J. Zhang et al., 2015).

MicroRNAs as potential biomarkers

A biomarker is a molecule that can be detected and quantified in a sample of body liquids, cells or tissues. The biomarker potentially says something about an underlying condition of the organism and therefore, is used for diagnostics and treatment planning (Califf, 2018; Vaishya et al., 2018). The significance of miRNAs as biomarkers are still uncertain but there are studies showing that miRNAs can be used in the diagnosis of several diseases. According to Vaishya et al. (2018), recent studies suggest that expression of biomolecules, such as miRNA, specifically changes during the development of T2DM, which means that miRNA potentially could be a biomarker for prognosis and diagnosis of the disease. MicroRNA can be isolated from cells, tissue and body fluids as serum, plasma, saliva or urine. According to Cao et al. (2019) circulating miRNAs are relatively stable under different storage conditions such as long-term room temperature storage and varying pH. To detect and measure miRNA different methods are used, for examples quantitative PCR (qPCR), microarrays or RNA sequencing (Lu & Rothenberg, 2018).

Quantitative reverse transcriptase PCR for microRNA analysis

A widely used method to profile miRNA is by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The method rely on converting RNA to cDNA by reverse transcriptase enzymes, followed by PCR amplification of cDNA and quantification by real time monitoring of generated PCR products from the reaction (Pritchard et al., 2012).

Although it is possible to extract miRNAs from multiple cell types and body fluids, there remains many challenges for accurate detection and quantification of these molecules. The main cause of these issues is due to the short length of these nucleotides. Because the length of a miRNA is typically 22 nucleotides long, the use of traditional PCR primers is insufficient, as these are about the same length (Lu & Rothenberg, 2018).

After isolation of total RNA from the sample, RNA molecules are translated to cDNA by the Reverse Transcriptase enzyme (Lu & Rothenberg, 2018). For this step, the miRNA must be extended using one of two strategies, either a stem-loop primer (illustrated in Figure 4) or by adding a poly A tail, and then using a poly T primer (illustrated in Figure 5).

In the first strategy, the stem-loop primers are specific to the 3′ end of the miRNA. After reverse transcriptase have completed synthesis of a complementary miRNA cDNA strand from the starting point of the stem-loop primer, the PCR can be performed (Pritchard et al., 2012). Cycle by cycle, amplicons are generated using a miRNA-specific forward primer. As DNA polymerase proceeds along the cDNA template, a hybridized TaqMan probe is cleaved so the quencher is freed from reporter fluorescent dye, resulting in light emission.

Using the second strategy, when extending the 3' end of the miRNA with a poly A tail, the reverse transcription can be done using a Poly T primer (Figure 5) (Lu & Rothenberg, 2018). This primer has a universal sequence (Tag) appended at its 5'end, which becomes binding site for the universal reverse primer during qPCR. The forward primer is then specific to the mRNA and miRNA itself.

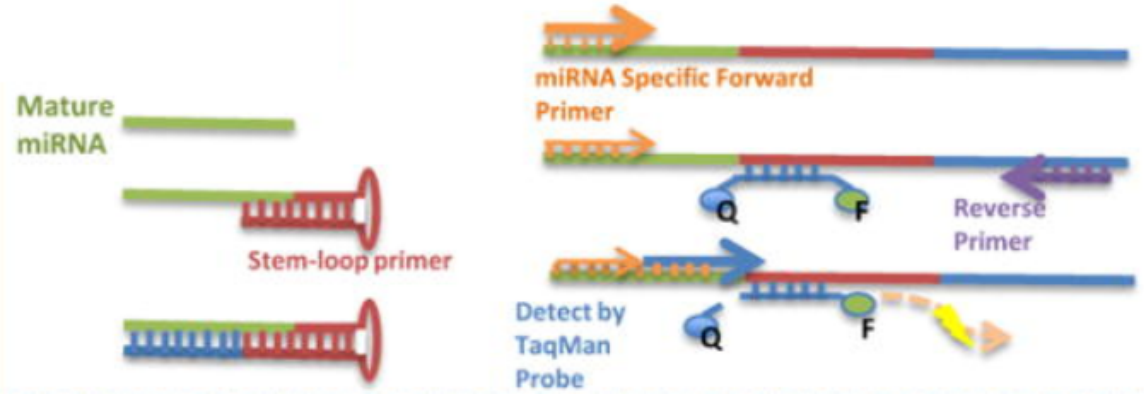

Figure 4: Reverse transcription is first done with a stem-loop primer that is specific to the 3'end of the miRNA. PCR is then performed with a miRNA specific forward primer. As DNA polymerase proceeds along the template, a probe is eventually hydrolysed, so the quencher is freed from the fluorescent dye, and light is emitted (Pritchard et al., 2012).

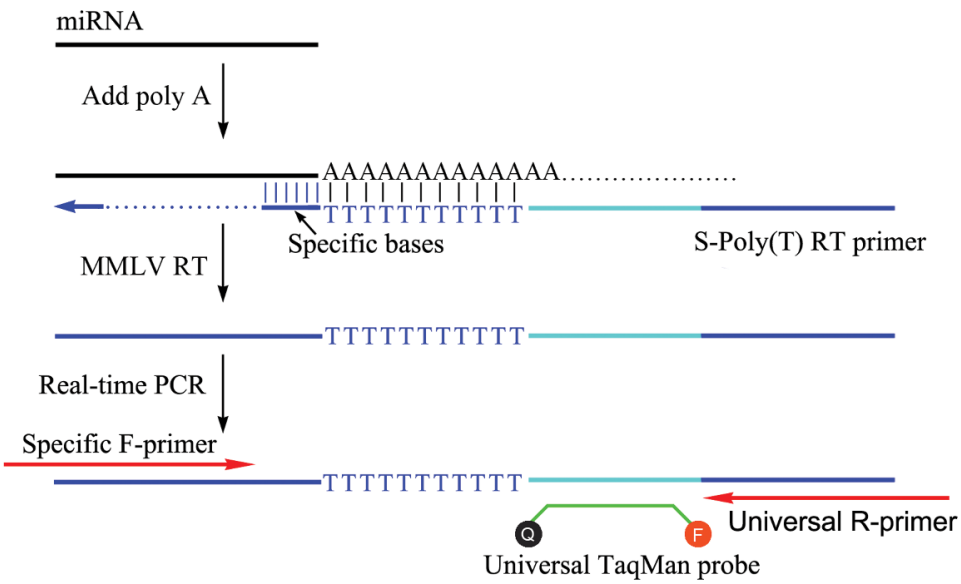

Figure 5: "Schematic representation of the S-Poly(T) method for miRNA quantification" (Kang et al., 2012). Reverse transcription is first done with polyadenylation and a tagged poly T primer at the 3'end of the miRNA. Then PCR is performed with a miRNA specific forward primer and a reverse primer specific to the universal sequence appended at the 5'end of the poly T primer.

In miRNA profiling, the qRT-PCR approach is usually carried out in highly parallel and high-throughput form, so that multiple reactions are measuring different miRNAs under the same reaction conditions. For this purpose, commercially available plates and cards can be applied to examine miRNAs that regulates a pathway of interest or a broader coverage (Pritchard et al., 2012).

Diabetes mellitus and diabetic kidney disease

Diabetes mellitus is a metabolic disease where there is a defect in insulin secretion, insulin action or both. Diabetes patients are usually categorized in type 1 or type 2 (T1DM or T2DM) (American Diabetes Association, 2013). T1DM accounts for 5-10% of patients with diabetes and originates from an autoimmune mediated destruction of the insulin producing β-cells of the pancreas. With this type of diabetes, the individuals usually have hyperglycemia due to absolute insulin deficiency and need insulin injections to survive. Diabetes mellitus type 1 is diagnosed early in life, childhood, but can also be found at any age. Insulin deficiency can be caused from multiple genetic predispositions and some environmental factors are also

involved, but the pathogenesis is still inadequately defined. Diabetes mellitus type 2 accounts for 90-95% of patients with diabetes. Individuals with T2DM do not have absolute insulin deficiency like type 1, but they have insulin resistance with relative insulin deficiency. Therefore, individuals with type 2 diabetes, often, does not initially and throughout their lifetime need insulin treatment to survive. Thus, the individuals with type 2 diabetes are often undiagnosed and have few classical symptoms for diabetes.

Diabetes patients have the misfortune not to be able to balance their blood sugar as effectively as healthy individuals, leading to high blood sugar more often (American Diabetes Association, 2013). Having high blood sugar or hyperglycemia over time, can impact the body and cause damage, dysfunctions and failure of different organs. The organs particularly most vulnerable are the kidneys, eyes, nerves, heart and blood vessels.

One of the most common complication from diabetes is kidney disease (Regmi et al., 2019). One in four adults with diabetes have kidney disease, resulting in damage to the functions of the kidneys (NIDDKD, 2017). Kidney disease caused by diabetes is often alternatively referred to as chronic kidney disease (CKD) or diabetic nephropathy (DN), but commonly known as diabetic kidney disease (DKD) (NIDDKD, 2017). There are various criteria for diagnosing individuals with diabetic kidney disease. GFR must be below 60 mL/min/1.73 m^2 and/or increased urinary albumin excretion (UAE) for at least three months (Nascimento & Domingueti, 2019). Increased UAE is defined as an albumin-to-creatinine ratio (ACR) $\geq$ 30 mg/g or albumin levels $\geq$ 30 mg in twenty-four-hour urinary protein (Nascimento & Domingueti, 2019). Assessment of GFR and UAE is important for early diagnosis and enables the further categorization of chronic kidney disease. However, there are still not any minimally invasive biomarker on the market that are highly specific and sensitive enough to catch DKD at an early stage (Regmi et al., 2019).

Diabetic kidney disease is a lasting damage to the kidneys and usually a permanent one (Regmi et al., 2019). The kidneys function efficiency of filtering waste and extra fluid out of the blood is decreased. DKD consist of 5 stages, from stage 1 with mild damage to stage 5 with kidney failure called end stage kidney disease (ESKD) (Regmi et al., 2019). If the patient reaches complete kidney failure (ESKD), they will require dialysis or renal transplantation to survive.

Aims of study

Diabetes mellitus is a widespread disease and many patients develop kidney disease because of diabetes. Today's preferred indicators for kidney function and glomerular injury is eGFR, albumin-creatinine ratio and proteinuria (albuminuria). Though, these are predictors of renal function decline, they lack the ability of early diagnosis of diabetic kidney disease (DKD) and to discriminate well between different disease states. Since the established biomarkers fall short, studies are now looking at miRNAs as potential analytes of DKD, both for diagnostic and therapeutic purposes. Differential expression of miRNAs correlated to different disorders has been studied for many years, but miRNA as potential biomarkers of DKD is a relatively new field and despite several research studies reported, there is still much uncertainty regarding differential expression of miRNAs correlated to DKD. Few, if any research studies, have been able to find miRNAs that are associated to DKD with certainty.

Therefore, a literature study is performed to make an overview on the status of this research field and to comment on miRNAs as potential biomarkers of DKD.

Material and Method

PubMed (https://www.ncbi.nlm.nih.gov/pmc/) is the database used in this literature study. PubMed consists of biomedical literature from MEDLINE, journals and online books. We searched for articles examining the use of urinary, plasma and serum levels of miRNA in diabetic patients with kidney complications. We selected articles only using human material to examine the differential expression of miRNA in DKD. Keywords that were used include "miRNA", "microRNA", "diabetes", "kidney disease", "biomarker" and "qPCR", often in combination. There were a lot of hits on these keywords. As an example, using the combination of words: "miRNA AND diabetes AND kidney" gave 520 results in PubMed, whereas "miRNA AND diabetes AND kidney AND biomarker" gave 149 result in the database (15.05.20). These 149 articles were further screened first by reading titles and abstracts and articles considered relevant were selected for further analysis. Some additional articles were found in the reference list from other papers about miRNA. Articles not including analysis of either urinary or circulating miRNAs in human samples, were rejected. Other selection criteria were that the study population had to be diagnosed with diabetic mellitus (type 1 and/or 2) as well as kidney disease. The literature searches resulted in both review- and original articles, and only original articles were used in the result chapter overviewing the original research results found.

A total of 21 articles were found relevant in the literature search, where 7 had limited online access with only abstracts available. With both limited library access and time-schedule, not all articles were studied further. A selection of 7 open access online articles were studied more comprehensively, to get a deeper understanding of how these studies were conducted. Of the remaining articles, only methodical information and scientific findings were obtained and reported in this overview.

Result

The literature search gave 149 results in the database, 21 of these were considered as relevant research papers investigating miRNAs as potential biomarkers of DKD - which are represented in table 1. Out of these, 7 articles were not available, and the information from these research results is therefore extracted from the abstract. However, these limited access articles may contain more information pointing to even more discoveries and more precise methods used to find miRNA in diabetic kidney disease patients.

Tabell 1: Relevant research articles investigating differential expression of miRNAs in DKD.

AUTHOR	TITLE OF ARTICLE	PUBLISHED	REFERENCE NUMBER
Peng et al.	Urinary miR-29 Correlates With Albuminuria and Carotid Intima-Media Thickness in Type 2 Diabetes Patients	2013	1
Delić et al.	Urinary Exosomal miRNA Signature in Type II Diabetic Nephropathy Patients.	2016	2
Jia et al.	miRNAs in Urine Extracellular Vesicles as Predictors of Early-Stage Diabetic Nephropathy.	2016	3
Chien et al	Differential microRNA Profiles Predict Diabetic Nephropathy Progression in Taiwan	2016	4
Eissa et al.	Urinary Exosomal microRNA Panel Unravels Novel Biomarkers for Diagnosis of Type 2 Diabetic Kidney Disease	2016	Not available 5
Eissa, Matboli, & Bekhet	Clinical Verification of a Novel Urinary microRNA Panal: 133b, -342 and -30 as Biomarkers for Diabetic Nephropathy Identified by Bioinformatics Analysis	2016	Not available 6
Shao et al.	Changes of Serum Mir-217 and the Correlation With the Severity in Type 2 Diabetes Patients With Different Stages of Diabetic Kidney Disease	2017	7
Zhang et al.	Co-expression Analysis Among microRNAs, Long Non-Coding RNAs, and Messenger RNAs to Understand the Pathogenesis and Progression of Diabetic Kidney Disease at the Genetic Level	2017	8

Cardenas-Gonzalez et al.	Identification, Confirmation, and Replication of Novel Urinary MicroRNA Biomarkers in Lupus Nephritis and Diabetic Nephropathy.	2017	9
Yang et al.	Microribonucleic acid-192 as a specific biomarker for the early diagnosis of diabetic kidney disease.	2017	10
Xie et al.	Urinary Exosomal MicroRNA Profiling in Incipient Type 2 Diabetic Kidney Disease	2017	11
El-Samahy et al.	Urinary miRNA-377 and miRNA-216a as Biomarkers of Nephropathy and Subclinical Atherosclerotic Risk in Pediatric Patients With Type 1 Diabetes	2017	Not available 12
Milas et al.	Deregulated Profiles of Urinary microRNAs May Explain Podocyte Injury and Proximal Tubule Dysfunction in Normoalbuminuric Patients With Type 2 Diabetes Mellitus	2018	Not available 13
Li et al.	Potential Value of Urinary Exosome-Derived let-7c-5p in the Diagnosis and Progression of Type II Diabetic Nephropathy	2018	Not available 14
Ghai et al.	Genome-wide Profiling of Urinary Extracellular Vesicle microRNAs Associated With Diabetic Nephropathy in Type 1 Diabetes	2018	15
Beltrami et al.	Association of Elevated Urinary miR-126, miR-155, and miR-29b with Diabetic Kidney Disease	2018	16
Prabu et al.	MicroRNAs From Urinary Extracellular Vesicles Are Non-Invasive Early Biomarkers of Diabetic Nephropathy in Type 2 Diabetes Patients With the 'Asian Indian Phenotype'	2018	Not available 17
Fouad et al.	MicroRNA-21 as an Early Marker of Nephropathy in Patients with Type 1 Diabetes.	2019	18
Regmi et al.	Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case-Controlled Study and Bioinformatics Analysis.	2019	19
Zang et al.	Differential Expression of Urinary Exosomal MicroRNAs miR-21-5p and miR-30b-5p in Individuals with Diabetic Kidney Disease.	2019	20
Petrica et al.	MiRNA Expression Is Associated With Clinical Variables Related to Vascular Remodeling in the Kidney and the Brain in Type 2 Diabetes Mellitus Patients	2020	Not available 21

The articles, presented in table 1, have in common that they studied the expression of miRNAs in diabetes patients with kidney diseases focusing on the relevance of miRNAs as potential diagnostic non-invasive biomarkers. The presented articles are published between 2013 and 2020.

Different methodological design studying miRNA expression in diabetes patients with kidney disease

The population groups varied in size in the different studies, as did the number of analysed miRNAs. Human sample material used in these articles were serum, plasma or urine and in some studies, sample material was extracted from urinary extracellular vesicles (EV) others specified using urinary exosome. MicroRNA is quantified by qRT-PCR in all the studies and some of the studies have used microarray and NGS, prior to qPCR.

Further information on methodological design in the different papers, like type of sample material, diagnostic groups in focus, type of control groups, population size, laboratory methods and number of tested miRNAs are shown in table 2. This to emphasise and compare the methodological similarities and differences, to further discuss the weaknesses and strengths among the different studies.

Table 2: Methodological parameters utilized studying differential miRNA expression in DKD.

REF	STUDY POPULATION	POPULATION SIZE			SAMPLE MATERIAL	METHOD	NUMBER OF TESTED miRNAs
		DM	DKD	Healthy			
1	T2DM DN	41	42	-	Urine	qRT-PCR	3
2	T2DM DN	8	8	8	Urinary exosome	(Microarray) + qRT-PCR	(-) 14
3	T2DM DN	30	50	10	Urine EV	qRT-PCR	3
4	T2DM DN	12	38	-	Serum	qRT-PCR	5
5	T2DM DKD	180			Urinary exosome	qRT-PCR	(6) 3
6	T2DM DN	210			Urinary exosome	qRT-PCR	(3)
7	T2DM DKD	186	309	195	Serum	qRT-PCR	1
8	DKD	30	27	28	Plasma	(Microarray) + qRT-PCR	(88) 1
9	T1/T2DM DN	71	74	30	Urine	qRT-PCR	(2402) 12
10	DKD	92	138	53	Urine/ Serum	qRT-PCR	2
11	T2DM DKD	5	5	-	Urinary exosome	Microarray + qRT-PCR	496
12	T1DM DN	50		50	Urine	qRT-PCR	2
13	T2DM DN	68		11	Urine	-	3
14	T2DM DN	20	20	15	Urinary exosomes	qRT-PCR	3
15	T1DM DN	Not specified			Urine EV	NGS + qRT-PCR	Not specified
16	DKD	62	89	41	Urine	(TaqManArray) + qRT-PCR	(754) 3

17	T2DM DN	160			Urine EV	-	73
18	T1DM DN	120	120	100	Plasma	qRT-PCR	1
19	T2DM DKD	50	42	25	Serum	(Microarray) + qRT-PCR	(190) 4
20	T2DM DKD	30	36 18 (not DM)	-	Urinary exosome	qRT-PCR	87
21	T2DM DKD	76		11	Urine/ Plasma	qRT-PCR	6

Abbreviations used in Table 1:

DM: *Normoalbuminuria and DM patients.*

DKD*: micro- and macroalbuminuria + DKD/CKD/DN.*

Healthy: *normal healthy individuals.*

EV*: extracellular vesicle*

The selected articles compared in table 2 had the studied patient group in common. They all focused on diabetes patients, whether it was T1DM or T2DM, that also had developed kidney disease (DKD in table 2). The variety of the studies utilized samples from DKD patients and re-tested them. Here the majority of selected research studies used the already established biomarkers to diagnose kidney disease; albumin-creatinine ratio, albuminuria and eGFR. Others also studied correlations to biopsy results, as this is the most accurate diagnostic test. By retesting the biomarkers of kidney disease, the studies show exactly how progressed the disease was, and many compared the miRNA expression with the quantitative results from the already established biomarkers.

To investigate expression of miRNA most studies divided the contestants according to their diagnosis, and the diversion was somewhat different from study to study. Some studies preferred to test only on DM patients with normal kidney functions and the test group was DM patients with kidney disease (DKD), making the DM patients the control group. Other studies analysed miRNA expression in; DM patients, the test group DKD and healthy individuals, aiming for both the healthy individuals and DM to be control groups. The individuals defined as healthy had to pass a range of criteria, like for example not having a secondary kidney disease or any type of diabetes mellitus (Yang et al., 2017). The last approach to analyse miRNA levels in contestants was to compare the sample results of DKD patients with healthy

individuals. Many of the studies divided the DKD patients according to the stage of kidney damage; normoalbuminuria, microalbuminuria and macroalbuminuria. This strategy additionally distinguished if the tested miRNAs showed differential expressional levels early or later on in the kidney disease development. In table 2 the DM patients divided into normoalbuminuria is sorted under DM, whereas the DM patients with micro- or macroalbuminuria are both sorted under DKD.

Comparing methodologies used in the different research studies, there is one preferred method to quantify miRNA expression in DKD patients, namely qRT-PCR. Some of the studies are analysing a pre-selected number of miRNAs by utilizing specific PCR-primers. While other studies do a much broader exploration and perform non-targeted miRNA profiling analysis using microarrays or NGS in advance of targeted quantitative PCR on selected miRNAs to be further assayed. These more extensive studies started out with a screening of many/all miRNAs in the sample, thereafter miRNAs found to be correlated with DKD in the initial screening, were further investigated with qRT-PCR. However, there are also studies that executes larger miRNA profiling by using only qRT-PCR. An example of this is demonstrated in article 9, where thousands of miRNAs are initially profiled by qRT-PCR in a pooled sample of 2402 miRNA in DN-patients. Thereafter, the 12 most differentially expressed miRNAs are further examined in a different and a larger study population. There are also articles that analyse only one miRNA, for example article 18 that quantifies miR-21. While other studies have tested on a small panel of selected miRNAs, such as a 3 miRNA panel in article 6. By utilizing different methods, the compared papers have tried to find which miRNAs might be best correlated to diabetic kidney disease and therefore can be used as a future diagnostic biomarker.

Also illustrated in table 2, the sample material used for miRNA analysis varies between studies. Approximately 75% (16 of 21) of the studies quantify miRNAs in urine. The remaining 25 % have chosen serum or plasma as sample material. Consequently, urine seem to be the preferred sample material when analysing miRNA expression in individuals with diabetic kidney disease. Several (9 of 21) explored urinary EV miRNA levels. According to Sun and Lerman (2019) extracellular vesicles are protective and help to increase the stability of the miRNA. Some studies used both urine and serum or plasma comparing miRNA expression in both sample materials. Like, for instance, article 10 that measures miR-192 in serum and urine.

More than two-thirds of the research studies have defined T2DM as a test population. As mentioned earlier, 90-95% of diabetic patients are diagnosed with type 2 diabetes and it is therefore natural that this group dominates in the study population.

miRNAs of significant different expressional levels in patients with diabetic kidney disease

Different miRNAs reported to be differentially expressed in samples of DKD patients are presented in table 3. The miRNAs have been sorted into the respective categories of up- or downregulated compared to miRNA levels in control samples. There is a wide variety of different miRNAs that have been analysed, where surprisingly few studies have analysed on the same miRNAs. Article 15 is the only study that does not specify their miRNA dysregulation as up- or downregulated, therefore not included in table 3 or 4.

Table 3: MicroRNAs upregulated in samples from individuals with diabetic kidney disease.

↑ UPREGULATED miRNAs ↑		
MicroRNA (miR-x)*	**SAMPLE MATERIAL**	**REFERENCE**
15b	Urinary exosome	5
21	Plasma	4
21	Urine	13, 18
21-5p	Urinary exosome	20
24-3p	Urine	17
27b-3p	Urine	17
29a	Serum	1, 4
29b	Urine	16
30a	Urine	6
34a	Urinary exosome	5
99b	Serum	19
122-5p	Serum	19
124	Urine	13
126	Urine	16
133b	Urine	6
150-5p	Urinary exosome	11
155	Urine	16
192	Urine EV**	3**, 10
194	Urine EV**	3
215	Urine EV**	3
217	Serum	7
342	Urine	6
362-3p	Urinary exosome	11
377	Urine	12
636	Urinary exosome	5
877-3p	Urinary exosome	11

**The miRNAs are presented only in numbers, all correct names are actually written "miR-x", were x is the number.* **EV: extracellular vesicles

Table 4: MicroRNAs downregulated in samples from individuals with diabetic kidney disease.

↓ DOWNREGULATED miRNA ↓		
MicroRNA (miR-x)*	**SAMPLE MATERIAL**	**REFERENCE**
15a-5p	Urinary exosomes	11
15b-5p	Urinary exosome (EV**)	14, (17)
20a	Serum	19
21-5p	Urinary exosome	20
29c-5p	Urinary exosome	14
30b-5p	Urinary exosome	20
30d-5p	Urinary exosome	2
30e-5p	Urinary exosome	2
125a	Urine/blood	21
125b-5p	Urinary exosome	20
126	Urine/blood	21
146a	Urine/blood	21
192	Serum/ Urine	10, 13, 21
216	Urine	12
223-3p	Plasma	8
486-5p	Serum	19
1915-3p	Urine	9
2861	Urine	9
4532	Urine	9

** The miRNAs are presented only in numbers, all correct names are actually written "miR-x", were x is the number.*

***EV: extracellular vesicle*

Several studies show that miR-192 is upregulated. In article 3, miR-192-expression is positively correlated with urinary albumin excretion rate. This means that miR-192 is up-regulated when albumin is excreted in the urine. Proteins like albumin should normally be excreted in small amounts in the urine. Excretion of larger amount may therefore indicate

kidney disease. In article 10, miR-192 was compared in serum and urine. The study shows that miR-192 assay of urine samples is more sensitive than when miR-192 is assayed in serum. Whereas miR-192 assayed in serum, on the other hand, is more specific. Interestingly, the study showed that miR-192 in urine was upregulated with progression of DKD and miR-192 in serum was downregulated with progression to DKD.

In article 18 and 20, miR-21 is reported to be upregulated in DKD patient samples. Article 1 showed miR-21 to be positively correlated with increased age, duration of diabetes, blood pressure, HbA1c and ACR. In article 18, miR-21 is reported to be upregulated in plasma and is suggested to serve as an early biomarker for DN even before detected microalbuminuria. The miR-21 levels were demonstrated to be significantly up-regulated in plasma of T1DM patients as compared to healthy persons, and miR-21 expression began to rise early in the first 5 years of the disease. The miR-21 seems to have a greater sensitivity and specificity to identify DN than ACR according to article 18.

Article 11, 14 and 17 report that miR-15 is downregulated in the studies. Two types of miR-15 have been found; miR-15a-5p and miR-15b-5p, both detected in urinary extracellular vesicles. Another miRNA that has been found downregulated in several studies is miR-30. Three types, miR-30b-5p, miR-30d-5p and miR-30e-5p, are detected in urinary exosomes in article 2 and 20.

Discussion

Methodological considerations of this literature study

A literature study has been performed as a method in this study. Ideally, we would write a more extended systematic literature study, but due to Covid-19 we were unable to achieve this. The time required to complete the thesis was shorter than expected, due to a non-productive project period in South Africa, and as library services at Western Norway University of Applied Sciences have been limited since 12th of March. Therefore, it became difficult to get access to as many articles as we wanted after the literature search, and our result is therefore more limited than it would be without Covid-19 pandemics. Of 21 selected articles, 14 articles were available to read in detail, as for the remaining 7 articles general information were obtained from the abstract. Then 7 available articles were selected and analysed more thoroughly than the others. This is due to limited time and resources. With better time and library services, all selected articles from the literature search would have been analysed in more depth and could have played a bigger role in the literature review.

Considerations of sample material when measuring miRNA expression in patients with diabetic kidney disease

In clinical analysis as well as in analysis for research, the results need to be trustworthy. Therefore, the stability of microRNAs must be taken to consideration. The articles that are reviewed in this study, all used either human plasma, serum or urine samples to identify miRNAs. As good markers that can quickly and easily predict kidney disease are missing today, further research, looking for new and better biomarkers in easily available sample material, is still necessary.

Approximately 75% of the reviewed articles have used urine as sample material in their study, as miRNAs are released by cells of the nephron and downstream in the urinary tract, reflecting the renal pathology (Cao et al., 2019). An advantage of using urinary miRNA as biomarkers is the non-invasive sampling and samples can easily be collected and sent to a test laboratory (Simpson et al., 2016). In both homeostatic and disease conditions, urine contains dissolved waste material that can be used as a biomarker for kidney disease (Sun & Lerman, 2019). According to Sun and Lerman it is still uncertainties regarding the validity of urinary miRNA

as biomarkers, still, an increasing number of studies have analysed miRNA in the urine attempting to detect and sub-classify kidney diseases. Simpson et al. claims that analysis of miRNAs performed in urine samples have been proved to be rather stable (Simpson et al., 2016). Concluding that relatively robust methods for quantification of urinary miRNAs have been established today.

Human blood plasma and serum are a challenging sample material of miRNA analysis because there are high levels of endogenous RNase activity and multiple sources of variation. Endogenous plasma miRNAs seem to be protected against RNase but if extracted miRNAs are spiked back into blood plasma degradation is observed within seconds. Pritchard et al. (2012) consequently suggests that RNA extraction methods must inactivate RNase completely and rapidly. Simpson et al. (2016) claims that the association of miRNAs with AGO2 proteins protects miRNAs from degradation in RNase-rich biological fluids such as blood. This is because extracellular miRNAs binding with AGO proteins result in the formation of ribonucleoprotein complexes with RISC that stabilise these molecules (Simpson et al., 2016). Due to this knowledge, and also as a disadvantage, when performing the extraction method of plasma or serum specimen, a complete and rapid RNase inactivation is required. The studies choosing these sample materials also had to deal with other pre-analytic factors, which may have had an impact on their results. Centrifugation conditions, cell lysis, and white blood cell counts are all plasma associated variables that can influence the nature and quantity of the miRNAs (Pritchard et al., 2012).

In several studies' extracellular vesicles, specially exosome, were extracted from urine to quantify the miRNA content. As so many studies found exosomes interesting for quantitation of miRNAs, urinary exosomes could possibly become the preferred sample material for clinical testing. Cells are sending out regulative miRNAs to other cells as cargo in exosomes, and therefore isolating and analysing the content of these extracellular vesicles may be of great information. Exosome miRNAs are also considered more stable compared to free circulating miRNAs since they are protected from endogenous RNase activity (Delić et al., 2016). If the research eventually finds that exosome miRNAs are representing the inner state of the kidney better than the free miRNAs, then the analysis procedures will be more resource demanding than analysing free circulating miRNAs. Analysing exosome miRNAs starts with isolating the

exosomes. The preferred method of extracting exosomes is by ultracentrifugation, where other methods are size-exclusion chromatography (SEC)-based method and commercial kits (Ghai et al., 2018). Jia et. al and Xia et. al both centrifugated urine samples two times in 70 minutes to isolate the exosome. This is a time- consuming additional step that will demand more resources at a higher cost. The method may be more trouble than useful in standard laboratories if free miRNAs are just as good indicators of diabetic kidney disease as exosome miRNAs.

As illustrated in table 3 and 4, one specific miRNA can be observed that has been found both upregulated and downregulated in DKD individuals, miR-192. miR-192 is a microRNA specifically expressed in the kidney (Yang et.al). Jia et.al quantified miR-192 from the extracellular vesicle in urine, while Yang et.al measured mir-192 in both serum and urine (Jia et al., 2016; Yang et al., 2017). Both studies found that miR-192 in urine was significantly increased in DKD individuals, in the contrary serum miR-192 were found to be downregulated. Interesting findings from both studies showed that increased level of urine miR-192 was found to be highest in DM microalbuminuric groups and lower in macroalbuminuric groups. Yang et.al also reported that the combination of serum and urine miR-192 analysis gave higher specificity and thus lowering the misdiagnosis rate. Even so, Yang et.al concluded that urinary miR-192 was a better single biomarker of DKD than serum miR-192. Meaning both Jia and Yang et.al came to the same conclusion that urinary miR-192 may be a valuable early diagnostic tool for DKD.

It should also be mentioned that although these two articles had significant results. Earlier articles from 2013 found that miR-192 is not up-regulated in urine samples of DKD patients (Delić et al., 2016).

Defining different populations to be compared have an impact on study results

The different research studies can be categorized according to three main strategies regarding the populations dividing the tested individuals, as shown in table 2. The different populations to be compared were: 1) DKD and healthy, 2) DM and DKD, 3) DM, DKD and healthy. This is interesting because it complicates the interpretation of different study results a little.

Regarding strategy 1), testing DKD patients to be compared with healthy individuals is opening for the chance that the result may be from the diabetes diagnosis. This strategy does not distinguish the dysregulated miRNA expression from the DM and the kidney diagnosis. Consequently, there is a risk that miRNAs observed to be differentially expressed is related to pathogenesis of diabetes mellitus and is not specific of the kidney disease.

Testing strategy 2) distinguish DM against the kidney disease diagnosis, by comparing miRNA expressional levels in these groups. Although, using DM patients as the control group, is non-informative weather miRNA results can be used as a biomarker for diagnosing non-diabetic kidney disease. Test strategy 3) differentiate the result of DKD patients from DM and healthy individuals, being most informative on miRNAs as possible biomarkers of diabetic kidney disease. Test strategy 3) shows when the differential expression of miRNA is not correlated to diabetic pathogenesis because the sample of both DM and healthy individuals are analysed to be compared. Being the most resource demanding strategy, testing DM, DKD and healthy individuals is the best way to differentiate miRNA expression profiles from DKD patients and non-diabetic individuals with kidney complications. Additionally, one study had a fourth strategy: 4) comparing DM, DKD, and non-diabetic kidney disease. In that way miRNAs are tested as biomarkers both of non-diabetic kidney disease and DKD.

Choice of quantitative methods analysing miRNA levels

As shown earlier, the field of research use separate strategies of miRNA screening but are heavily depending on qRT-PCR for the quantitative determination of miRNAs. Though these PCR-techniques have proved to be sensitive and specific, they include many preparatory steps, which increases the risk of sample contamination.

Unlike PCR, the procedure of microarrays is rather uncomplicated, and can also be used in relative quantitative measurements. With microarrays it is possible to directly label the total RNA in a sample and steps like separation or amplification which may induce sample manipulation, is removed (Wang et al., 2007). When fluorescent labelled miRNAs hybridize with capture probes located on the array multiple individual miRNAs are quantified in parallel. However, the sequence specificity of the capture probes varies, and therefore cross-hybridization of certain miRNAs is unavoidable. Although this is mostly observed for highly homologous miRNAs, that may only differ by a purine to purine or a pyrimidine to pyrimidine

substitution, it generally limits the methods specificity. Thus, miRNA microarrays are best used for comparing relative abundance of miRNAs between two states and cannot determine absolute quantification (Pritchard et al., 2012).

Quantifying miRNAs can also be done by NGS. The procedure starts with preparing a cDNA library from an RNA sample (Pritchard et al., 2012). This step may also include a PCR amplification. Next, millions of individual cDNA molecules are sequenced, in a "massively parallel" sequencer. At last bioinformatical analysis of the sequence reads can identify both known and novel miRNAs. Here, the total number of sequences reads for a given miRNA is an estimate of relative abundance of this miRNA in the sample. This means that comparisons between samples with varying distribution of miRNA expression, are currently challenging for NGS (Pritchard et al., 2012).

One can argue that all the studies presented in this literature study, have chosen proper ways to quantitatively determine and compare miRNA expression, as all the studies applies qRT-PCR to examine the microRNAs of interest. The method generally has the widest dynamic range and holds highest accuracy (Pritchard et al., 2012). When comparing if an expression level in a DKD patient is significantly different from a control group, it is crucial that the quantitative measurement is as accurate as possible and applying one of the other methods for this purpose, may not have produced such reliable results.

Many miRNAs have regulatory roles in both diabetic kidney disease and non-diabetic kidney disease individuals and some miRNAs found to be differentially expressed therefore have poor specificity. According to Cao et al. (2019) many studies have used urinary miRNA "biomarker panels", that are several miRNAs measured in combination, to optimize diagnostic sensitivity and specificity. Article 5 is an example of a study that use several miRNAs instead of one. The study uses a 6 miRNA panel in urine pellet and exosome, and select the most significant up-regulated miRNAs in DKD. Exosomal miR-15b, miR-34a and miR-636 were measured by qPCR used to evaluate their usefulness as novel urine biomarkers for diagnosing diabetic kidney disease in a population of 180 participants. In article 6, which uses a 3 miRNA panel, the purpose was to identify a new miRNA panel of DN.

Are microRNAs relevant biomarkers of diabetic kidney disease?

The miRNAs are a relatively new group of biological molecules to be studied. Understanding more about their regulatory effects and their connections to diseases, is considered as highly interesting and relevant research in the medical field. This interest of miRNAs potential applies especially for renal diseases because the current biomarkers for these conditions, hold a few drawbacks.

The most common, already, established biomarkers to diagnose kidney diseases are creatinine-based eGFR measurement, albuminuria, and albumin/creatinine ratio (Regmi et al., 2019; Zang et al., 2019). As stated earlier the currently most important steps to prevent further progression of DKD is early treatment, therefor getting a diagnosis as early as possible have improved prognostic features. The conventional biomarkers are often not sensitive and specific enough for that purpose, only invasive diagnostic procedures, like biopsy analysis, can specifically tell the renal current status early on (Regmi et al., 2019).

There are different reasons for the limitation to these testing methods, that makes them less reliable and inconvenient when it comes to earlier and more detailed diagnosis of DKD. Patients with DKD without proteinuria can occur, and often in T2DM patients (Zang et al., 2019). In that way people with a GRF <30 mL/min/1.73 m^2 showing no signs of proteinuria, becomes diagnosed normoalbuminuric (Bolignano & Zoccali, 2017). For instance, diagnosis of DN rely heavily on detection of urinary microalbuminuria (Simpson et al., 2016). A reduced GFR is surveyed to be more common among diabetic patients than non-diabetic patients (Bolignano & Zoccali, 2017). These patients are therefore in even higher risk of developing kidney disease further without an early diagnosis, causing renal dysfunction and progression to ESKD. Other interferences can be extremes of body mass index with the creatinine based GFR formula (Zang et al., 2019). Also some individuals have glomerular hyperfiltration in the early stages of DKD, causing more trouble for the GFR formula (Zang et al., 2019).

In this literature study we found several studies that indicates correlation between different types of miRNA and diabetic kidney disease. The advantages of possibly using miRNAs as biomarkers of DKD are more information than the conventional biomarkers and the non-invasive testing procedures as urinary samples are easily handled. New technologies make the

extraction of total miRNA from different body fluids easier, while quantitative methods to measure miRNA expression have become more standardized. However, the methodology of isolating RNA, to synthesize cDNA, and the extra customized PCRs of the short miRNAs, are still resource intensive to be considered as diagnostic analysis to be performed in the routine medical laboratories. Introducing the additional procedures of isolating the exosomes, complicates the procedures even more, which to our opinion is challenging for a method to be considered as routine medical laboratory analysis.

Summarized, no miRNA biomarker assays have been established in diagnostics of DKD yet. The studies in these articles aimed to use miRNAs as new biomarkers, in non-invasive sample material. Researchers have only started to investigate the potential use of miRNAs as biomarkers diagnosing diabetic kidney disease. Further developing the methodology, the miRNAs have a potential to decrease the financially and deathly burdening of the world from diabetic kidney disease.

References

American Diabetes Association. (2013). Diagnosis and classification of diabetes mellitus. *Diabetes Care*, *36 Suppl 1*, S67-74. https://doi.org/10.2337/dc13-S067

Badal, S. S., & Danesh, F. R. (2014). New insights into molecular mechanisms of diabetic kidney disease. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, *63*(2 Suppl 2), S63-83. https://doi.org/10.1053/j.ajkd.2013.10.047

Beltrami, C., Simpson, K., Jesky, M., Wonnacott, A., Carrington, C., Holmans, P., Newbury, L., Jenkins, R., Ashdown, T., Dayan, C., Satchell, S., Corish, P., Cockwell, P., Fraser, D., & Bowen, T. (2018). Association of Elevated Urinary miR-126, miR-155, and miR-29b with Diabetic Kidney Disease. *The American Journal of Pathology*, *188*(9), 1982–1992. https://doi.org/10.1016/j.ajpath.2018.06.006

Bolignano, D., & Zoccali, C. (2017). Non-proteinuric rather than proteinuric renal diseases are the leading cause of end-stage kidney disease. *Nephrology Dialysis Transplantation*, *32*(suppl_2), ii194–ii199. https://doi.org/10.1093/ndt/gfw440

Califf, R. M. (2018). Biomarker definitions and their applications. *Experimental Biology and Medicine (Maywood, N.J.)*, *243*(3), 213–221. https://doi.org/10.1177/1535370217750088

Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., & Croce, C. M. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15524–15529. https://doi.org/10.1073/pnas.242606799

Cao, Q., Chen, X.-M., Huang, C., & Pollock, C. A. (2019). MicroRNA as novel biomarkers and therapeutic targets in diabetic kidney disease: An update. *FASEB BioAdvances*, *1*(6), 375–388. https://doi.org/10.1096/fba.2018-00064

Cardenas-Gonzalez, M., Srivastava, A., Pavkovic, M., Bijol, V., Rennke, H. G., Stillman, I. E., Zhang, X., Parikh, S., Rovin, B. H., Afkarian, M., de Boer, I. H., Himmelfarb, J., Waikar, S. S., & Vaidya, V. S. (2017). Identification, Confirmation, and Replication of Novel Urinary MicroRNA Biomarkers in Lupus Nephritis and Diabetic Nephropathy. *Clinical Chemistry*, *63*(9), 1515–1526. https://doi.org/10.1373/clinchem.2017.274175

Chien, H.-Y., Chen, C.-Y., Chiu, Y.-H., Lin, Y.-C., & Li, W.-C. (2016). Differential

microRNA Profiles Predict Diabetic Nephropathy Progression in Taiwan. *International Journal of Medical Sciences*, *13*(6), 457–465. https://doi.org/10.7150/ijms.15548

Delić, D., Eisele, C., Schmid, R., Baum, P., Wiech, F., Gerl, M., Zimdahl, H., Pullen, S. S., & Urquhart, R. (2016). Urinary Exosomal miRNA Signature in Type II Diabetic Nephropathy Patients. *PloS One*, *11*(3), e0150154. https://doi.org/10.1371/journal.pone.0150154

Eissa, S., Matboli, M., Aboushahba, R., Bekhet, M. M., & Soliman, Y. (2016). Urinary exosomal microRNA panel unravels novel biomarkers for diagnosis of type 2 diabetic kidney disease. *Journal of Diabetes and Its Complications*, *30*(8), 1585–1592. https://doi.org/10.1016/j.jdiacomp.2016.07.012

Eissa, S., Matboli, M., & Bekhet, M. M. (2016). Clinical verification of a novel urinary microRNA panal: 133b, -342 and -30 as biomarkers for diabetic nephropathy identified by bioinformatics analysis. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, *83*, 92–99. https://doi.org/10.1016/j.biopha.2016.06.018

El-Samahy, M. H., Adly, A. A., Elhenawy, Y. I., Ismail, E. A., Pessar, S. A., Mowafy, M. E.-S., Saad, M. S., & Mohammed, H. H. (2018). Urinary miRNA-377 and miRNA-216a as biomarkers of nephropathy and subclinical atherosclerotic risk in pediatric patients with type 1 diabetes. *Journal of Diabetes and Its Complications*, *32*(2), 185–192. https://doi.org/10.1016/j.jdiacomp.2017.10.014

Esquela-Kerscher, A. (2014). The lin-4 microRNA: The ultimate micromanager. *Cell Cycle (Georgetown, Tex.)*, *13*(7), 1060–1061. https://doi.org/10.4161/cc.28384

Fouad, M., Salem, I., Elhefnawy, K., Raafat, N., & Faisal, A. (2020). MicroRNA-21 as an Early Marker of Nephropathy in Patients with Type 1 Diabetes. *Indian Journal of Nephrology*, *30*(1), 21–25. https://doi.org/10.4103/ijn.IJN_80_19

Ghai, V., Wu, X., Bheda-Malge, A., Argyropoulos, C. P., Bernardo, J. F., Orchard, T., Galas, D., & Wang, K. (2018). Genome-wide Profiling of Urinary Extracellular Vesicle microRNAs Associated With Diabetic Nephropathy in Type 1 Diabetes. *Kidney International Reports*, *3*(3), 555–572. https://doi.org/10.1016/j.ekir.2017.11.019

Griffiths-Jones, S., Grocock, R. J., Dongen, S. van, Bateman, A., & Enright, A. J. (2006). miRBase: MicroRNA sequences, targets and gene nomenclature. *Oxford University Press*, *34*(Nucleic Acids Research). https://doi.org/10.1093/nar/gkj112

Jia, Y., Guan, M., Zheng, Z., Zhang, Q., Tang, C., Xu, W., Xiao, Z., Wang, L., & Xue, Y.

(2016). MiRNAs in Urine Extracellular Vesicles as Predictors of Early-Stage Diabetic Nephropathy. *Journal of Diabetes Research*, *2016*, 7932765. https://doi.org/10.1155/2016/7932765

Kang, K., Zhang, X., Liu, H., Wang, Z., Zhong, J., Huang, Z., Peng, X., Zeng, Y., Wang, Y., Yang, Y., Luo, J., & Gou, D. (2012). A Novel Real-Time PCR Assay of microRNAs Using S-Poly(T), a Specific Oligo(dT) Reverse Transcription Primer with Excellent Sensitivity and Specificity. *PLOS ONE*, *7*(11), e48536. https://doi.org/10.1371/journal.pone.0048536

Kato, M., & Natarajan, R. (2014). Diabetic nephropathy—Emerging epigenetic mechanisms. *Nature Reviews. Nephrology*, *10*(9), 517–530. https://doi.org/10.1038/nrneph.2014.116

Li, W., Yang, S., Qiao, R., & Zhang, J. (2018). Potential Value of Urinary Exosome-Derived let-7c-5p in the Diagnosis and Progression of Type II Diabetic Nephropathy. *Clinical Laboratory*, *64*(5), 709–718. https://doi.org/10.7754/Clin.Lab.2018.171031

Linse, K. D. (2013, April 11). *How can MicroRNAs be descriped?* Bio-Synthesis, Inc. https://blog-biosyn.com/2013/04/11/how-can-micrornas-be-descriped/

Lu, T. X., & Rothenberg, M. E. (2018). MicroRNA. *The Journal of Allergy and Clinical Immunology*, *141*(4), 1202–1207. https://doi.org/10.1016/j.jaci.2017.08.034

Milas, O., Gadalean, F., Vlad, A., Dumitrascu, V., Gluhovschi, C., Gluhovschi, G., Velciov, S., Popescu, R., Bob, F., Matusz, P., Pusztai, A.-M., Cretu, O. M., Secara, A., Simulescu, A., Ursoniu, S., Vlad, D., & Petrica, L. (2018). Deregulated profiles of urinary microRNAs may explain podocyte injury and proximal tubule dysfunction in normoalbuminuric patients with type 2 diabetes mellitus. *Journal of Investigative Medicine: The Official Publication of the American Federation for Clinical Research*, *66*(4), 747–754. https://doi.org/10.1136/jim-2017-000556

Nascimento, L. R. do, & Domingueti, C. P. (2019). MicroRNAs: New biomarkers and promising therapeutic targets for diabetic kidney disease. *Jornal Brasileiro De Nefrologia: 'Orgao Oficial De Sociedades Brasileira E Latino-Americana De Nefrologia*, *41*(3), 412–422. https://doi.org/10.1590/2175-8239-JBN-2018-0165

(NIDDKD) National Institute of Diabetes and Digestive and Kidney Diseases. (2017, February). *Diabetic Kidney disease*. U.S. Department of Health and Human Services. https://www.niddk.nih.gov/health-information/diabetes/overview/preventing-problems/diabetic-kidney-disease

Peng, H., Zhong, M., Zhao, W., Wang, C., Zhang, J., Liu, X., Li, Y., Paudel, S. D., Wang, Q., & Lou, T. (2013). Urinary miR-29 correlates with albuminuria and carotid intima-media thickness in type 2 diabetes patients. *PloS One*, *8*(12), e82607. https://doi.org/10.1371/journal.pone.0082607

Petrica, L., Pusztai, A.-M., Vlad, M., Vlad, A., Gadalean, F., Dumitrascu, V., Vlad, D., Velciov, S., Gluhovschi, C., Bob, F., Ursoniu, S., Petrica, M., Matusz, P., Cretu, O., Radu, D., Milas, O., Secara, A., Simulescu, A., Popescu, R., & Jianu, D. C. (2020). MiRNA Expression is Associated with Clinical Variables Related to Vascular Remodeling in the Kidney and the Brain in Type 2 Diabetes Mellitus Patients. *Endocrine Research*, *45*(2), 119–130. https://doi.org/10.1080/07435800.2019.1690505

Prabu, P., Rome, S., Sathishkumar, C., Gastebois, C., Meugnier, E., Mohan, V., & Balasubramanyam, M. (2019). MicroRNAs from urinary extracellular vesicles are non-invasive early biomarkers of diabetic nephropathy in type 2 diabetes patients with the 'Asian Indian phenotype'. *Diabetes & Metabolism*, *45*(3), 276–285. https://doi.org/10.1016/j.diabet.2018.08.004

Pritchard, C. C., Cheng, H. H., & Tewari, M. (2012). MicroRNA profiling: Approaches and considerations. *Nature Reviews. Genetics*, *13*(5), 358–369. https://doi.org/10.1038/nrg3198

Regmi, A., Liu, G., Zhong, X., Hu, S., Ma, R., Gou, L., Zafar, M. I., & Chen, L. (2019). Evaluation of Serum microRNAs in Patients with Diabetic Kidney Disease: A Nested Case-Controlled Study and Bioinformatics Analysis. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *25*, 1699–1708. https://doi.org/10.12659/MSM.913265

Shao, Y., Ren, H., Lv, C., Ma, X., Wu, C., & Wang, Q. (2017). Changes of serum Mir-217 and the correlation with the severity in type 2 diabetes patients with different stages of diabetic kidney disease. *Endocrine*, *55*(1), 130–138. https://doi.org/10.1007/s12020-016-1069-4

Simpson, K., Wonnacott, A., Fraser, D. J., & Bowen, T. (2016). MicroRNAs in Diabetic Nephropathy: From Biomarkers to Therapy. *Current Diabetes Reports*, *16*(3), 35. https://doi.org/10.1007/s11892-016-0724-8

Sun, I. O., & Lerman, L. O. (2019). Urinary microRNA in kidney disease: Utility and roles. *American Journal of Physiology. Renal Physiology*, *316*(5), F785–F793. https://doi.org/10.1152/ajprenal.00368.2018

Vaishya, S., Sarwade, R. D., & Seshadri, V. (2018). MicroRNA, Proteins, and Metabolites as Novel Biomarkers for Prediabetes, Diabetes, and Related Complications. *Frontiers in Endocrinology*, *9*, 180. https://doi.org/10.3389/fendo.2018.00180

Wang, H., Ach, R. A., & Curry, B. (2007). Direct and sensitive miRNA profiling from low-input total RNA. *RNA (New York, N.Y.)*, *13*(1), 151–159. https://doi.org/10.1261/rna.234507

Wright, M. W., & Bruford, E. A. (2011). Naming 'junk': Human non-protein coding RNA (ncRNA) gene nomenclature. *Human Genomics*, *5*(2), 90–98. https://doi.org/10.1186/1479-7364-5-2-90

Xie, Y., Jia, Y., Cuihua, X., Hu, F., Xue, M., & Xue, Y. (2017). Urinary Exosomal MicroRNA Profiling in Incipient Type 2 Diabetic Kidney Disease. *Journal of Diabetes Research*, *2017*, 6978984. https://doi.org/10.1155/2017/6978984

Yang, X., Liu, S., Zhang, R., Sun, B., Zhou, S., Chen, R., & Yu, P. (2017). Microribonucleic acid-192 as a specific biomarker for the early diagnosis of diabetic kidney disease. *Journal of Diabetes Investigation*. https://doi.org/10.1111/jdi.12753

Zang, J., Maxwell, A. P., Simpson, D. A., & McKay, G. J. (2019). Differential Expression of Urinary Exosomal MicroRNAs miR-21-5p and miR-30b-5p in Individuals with Diabetic Kidney Disease. *Scientific Reports*, *9*(1), 10900. https://doi.org/10.1038/s41598-019-47504-x

Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., & Mi, S. (2015). Exosome and exosomal microRNA: Trafficking, sorting, and function. *Genomics, Proteomics & Bioinformatics*, *13*(1), 17–24. https://doi.org/10.1016/j.gpb.2015.02.001

Zhang, L., Li, R., He, J., Yang, Q., Wu, Y., Huang, J., & Wu, B. (2017). Co-expression analysis among microRNAs, long non-coding RNAs, and messenger RNAs to understand the pathogenesis and progression of diabetic kidney disease at the genetic level. *Methods (San Diego, Calif.)*, *124*, 46–56. https://doi.org/10.1016/j.ymeth.2017.05.023

Definitions

ACR: albumin-to-creatinine ratio

AGO: Argonaute proteins

CKD: chronic kidney disease

DKD: diabetic kidney disease

DN: diabetic nephropathy

DM: diabetes mellitus

ESKD: end stage kidney disease

EV: extracellular vesicle

GFR: Glomerular filtration rate

NGS: Next Generation Sequencing

PCR: polymerase chain reaction

T1DM: type 1 Diabetes mellitus

T2DM: type 2 Diabetes mellitus

UAE: urinary albumin excretion