ABSTRACT

Diabetic nephropathy (DN) is a major microvascular complication of diabetes and the leading cause of ESRD, which can manifest despite tight glycemic control and various therapeutic interventions. Overt nephropathy is diagnosed when the albumin excretion rate (AER) persistently exceeds 300 mg in a 24-h urine collection. miRNA are currently recognized as potentially important regulators of genes involved in processes related to the development of diabetic nephropathy, and as such, represent viable targets for both clinical diagnostic strategies and therapeutic intervention. Recently, urine exosomes have been proposed as valuable tools for early diagnosis of DN ***(Musante et al., 2014)*** and also for predicting renal dysfunction and damage.

Aim: To select biomarker relevant to diabetic nephropathy from the databases and to evaluate its usefulness as a urine molecular marker for early diabetic nephropathy detection.

Results: Our results revealed that the urinary exosomal miR-133b can improve the diagnostic accuracy and miR-636 may be a useful predictive biomarker for the progression of diabetic nephropathy.

Conclusion: ***miRNA-133b and miRNA-636*** expression levels in urine sample is a potentially useful urinary biomarker for early diagnosis and prediction of progression of diabetic nephropathy.

Keywords:

Diabetic Nephropathy- MiRNA- Exosomes – miR-133b – miR-636

INTRODUCTION

Diabetes mellitus (DM) is the most common metabolic disorder worldwide. Because of population aging and increasing trends toward obesity and sedentary lifestyle, the number of affected individuals is increasing at a worrisome rate and is expected to double within the next 20 years ***(Guay et al., 2011)***. In year 2000 according to the World Health organization and United Nations, at least 171 million people worldwide suffered from diabetes and it is expected to reach 366 million by 2030. It is estimated that by year 2030, Egypt will have at least 8.6 million adults with diabetes ***(Arfa & El Din, 2010).***

Diabetic nephropathy (DN) is a major microvascular complication of diabetes and the leading cause of ESRD, which can manifest despite tight glycemic control and various therapeutic interventions ***(Declèves and Sharma, 2010).*** Diabetic nephropathy is a devastating chronic event that is characterized by persistent proteinuria, elevated arterial blood pressure, and decline in renal function. Overt nephropathy is diagnosed when the albumin excretion rate (AER) persistently exceeds 300 mg in a 24-h urine collection ***(Conserva et al., 2013).***

Epidemiological studies have demonstrated that diabetic nephropathy occurs in approximately one-third to one half of all patients with diabetes ***(Argyropoulos et al., 2013)***. Diabetic

nephropathy ranged from 6.7% in hospital outpatient clinics in Egypt to 46.3% in hospital inpatients in Egypt ***(Hamed et al., 2008).***

The current clinical marker of microalbuminuria as an early biomarker of diabetic nephropathy is fraught with controversy. Firstly, not all patients with microalbuminuria will progress. Several studies showed that although 29% of patients with type 1 diabetes mellitus (DM) develop microalbuminuria after 18 years of follow-up, only one third of those patients progress to persistent macroalbuminuria; in fact, one third of patients regress spontaneously to normoalbuminuria. Secondly, it is also a nonspecific marker, given that urinary albumin levels are upregulated in other kidney diseases (hypertensive nephrosclerosis, tubulointerstitial disease), and other organ diseases (retinopathy and congestive heart failure). Thus, there is substantial need for a biomarker that may enable detection of diabetic nephropathy at an earlier stage with higher accuracy, and one which may further improve our understanding of the pathophysiology and clinical management of diabetic patients ***(Mehta et al., 2013).***

Increased accumulation of extracellular matrix proteins and hypertrophy induced by transforming growth factor-β1 (TGF-β) in renal mesangial cells (MC) via both Smad transcription factors and E-box-dependent mechanisms are hallmark features of diabetic nephropathy. Although the post-transcriptional regulation of key genes has been implicated in

these events, details are not fully understood ***(Brosius et al., 2010)***.

Recent technological advances enable cost-effective investigations of functional risk factors for diabetic nephropathy including genetic, epigenetic, transcriptomic, proteomic and metabolomic pathways coupled with data from clinical observations and animal models of diabetic kidney disease. Analyzing integrated networks and pathways from rich and diverse data sources, often using systems biology-based approaches, is becoming an important component of diabetic nephropathy research ***(Afkarian et al., 2013).*** Improvements in bioinformatic analyses techniques is revealing novel findings from well designed, adequately powered studies for diabetic nephropathy ***(McBrien et al., 2012).***

Several miRNAs have been identified as having a physiological role in tissues in which diabetes complications occur. Whether these miRNAs are involved in the damage that occurs in diabetes is yet to be established. Given that each miRNA has the potential to regulate multiple genes, dysregulation of miRNAs would be certain to have impact on many biological processes that are of direct relevance to diabetes complications ***(Zhou et al., 2012).***

Recent attention has turned to the role of miRNAs in renal disease where Dicer knockout experiments have established the general importance of miRNA in normal renal

development and function ***(Kaucsár et al., 2010).*** Other studies have provided evidence linking prosclerotic factors and miRNAs to fibrosis and have provided the much needed impetus in this area with the promise of new approaches for the treatment of fibrosis. So miRNA are currently recognized as potentially important regulators of genes involved in processes related to the development of diabetic nephropathy, and as such, represent viable targets for both clinical diagnostic strategies and therapeutic intervention ***(Alvarez et al., 2013)***.

Aim of the Work

To select biomarker relevant to diabetic nephropathy from the databases and to evaluate its usefulness as a urine molecular marker for early diabetic nephropathy detection.

Chapter 1
DIABETIC NEPHROPATHY

Diabetic nephropathy is a major underlying cause of morbidity and mortality in both type 1 and type 2 diabetes mellitus. The all-cause mortality in patients with diabetic nephropathy is nearly 20–40 times higher than in patients without nephropathy. Nevertheless, diabetic nephropathy is the most common cause of end-stage renal disease worldwide. Over a lifetime, diabetic nephropathy occurs in approximately 30–35% of patients with type 1 and type 2 diabetes ***(Thomas and Karalliedde, 2015).*** The prevalence of diabetic nephropathy as a cause of end-stage renal disease in the Egyptian renal data system was evaluated during the period 1996-2001 for the prevalence of diabetic nephropathy, which gradually increased from 8.9% in 1996 to 14.5% in 2001 ***(Afifi et al., 2004)***.

Diabetic nephropathy or diabetic Kidney disease (DKD), also known as Kimmelstiel Wilson syndrome is a clinical syndrome characterized by the following:

- Persistent albuminuria (>300 mg/d or >200ug/min) that is confirmed on at least 2 occasions 3-6 months apart.
- Progressive decline in the glomerular filtration rate (GFR).
- Elevated arterial blood pressure ***(Giriraja et al., 2015).***

Molecular pathways involved in diabetic nephropathy:

1. Hemodynamic pathways:

Renin angiotensin aldosterone system has a crucial role in homeostatic control of tissue perfusion, arterial pressure and extracellular volume. Along with hemodynamic effects, RAAS is also involved in renal tissue cell infiltration and inflammation ***(Graciano et al., 2004).***

Another potent vasoconstrictor of the efferent arteriole is endothelin-1 (ET-1). ET-1 has various physiologic functions in the kidney that mimic RAS including mediating vasoconstriction and hence playing a role in hypertension, endothelial dysfunction, inflammation, and fibrosis ***(Benz and Amann, 2011).***

A growing body of evidence suggests that renin and its receptor [(Pro) renin receptor (PRR)] play a pivotal role in the pathogenesis of diabetic nephropathy. It has also been noted that PRR is involved in the development and progression of kidney disease during diabetes by enhancing the renal production of inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β), independent of the effects of renal angiotensin II (Ang II) ***(Matavelli et al., 2010).*** In addition, renin itself regulates the expression of TGF-β1 in mesangial cells through a receptor-mediated mechanism that in turn

stimulates plasminogen activator inhibitor-1 (PAI-1), fibronectin and collagen I ***(Hwang et al., 2006; Zhang et al., 2006).***

TGF-β1 is one of the major contributors to DN development. The abnormal architecture of podocytes contributes essentially to DN onset and subsequent complications (microalbuminuria/proteinuria, decrease in GFR, inflammation). TGF-β1 overexpression is responsible for the apoptosis of podocytes through interaction with specific receptors (Alk5 and Alk1). A signaling cascade involving Sad proteins and MAP kinases is thus initiated ***(Badshah et al., 2014; Montero et al., 2015).***

2. Metabolic pathways:

The hyperglycemia-induced metabolic derangements coupled with channeling of glucose intermediaries into polyol and hexosamine pathways lead to the activation of protein kinase C and the generation of advanced glycation endproducts (AGEs) and reactive oxygen species (ROS) ***(Kanwar et al., 2011)***.

A. Polyol pathway and oxidative stress

In the state of hyperglycemia, the activity of aldose reductase (AR) increases and then polyol pathway is activated, which will turn excessive intracellular glucose to sorbitol. Sorbitol can be further converted into fructose and its

metabolites ***(Yagihashi and Wada, 2005).*** The study has found that excessive ROS inhibits activity of GAPDH in the state of hyperglycemia, resulting in the accumulation of 6 – phosphate glucose, thus activating the polyol pathway. The increase of AR inhibits glutathione reductase (GSR), which will cause the decrease of major intracellular antioxidant reduced glutathione GSH ***(Srivastava et al., 2005).*** It can exacerbate the oxidative stress. Finally, the end product of the polyol pathway, fructose, has also recently emerged as a potential nephrotoxin. In a diabetic murine model, endogenous production of fructose through the polyol pathway led to increased proteinuria, reduced GFR, and increased glomerular and proximal tubular injury when compared to mice with lower levels of endogenous fructose ***(Lanaspa et al., 2014).***

B. AGE pathway and oxidative stress

Oxidative stress is one of the major factors in the pathogenesis of diabetic complications; particularly, diabetic nephropathy and hyperglycemia are important sources of reactive oxygen species (ROS) ***(Ha et al., 2008)***. Advanced glycation end-products (AGEs), resulting from binding of sugars to proteins, lipids and nucleic acids by nonenzymatic reactions have been implicated as key pathogenic factors in initiation and progression of diabetic complications ***(Singh et al., 2001)***. One of the important pathways through which AGEs may exert their pathological effects is via interaction with the

receptor for advanced glycation end product (RAGE) ***(Stern et al., 2002).***

For example, AGE modifies both laminin and type IV collagen and was shown to increase the permeability of the glomerular basement membrane (GBM). Additionally increased concentrations of AGE are known to dose-dependently increase expression of fibronectin and collagen types I and IV which are thought to lead to increased density and expansion of the extracellular matrix in the kidney ***(Forbes et al., 2003)***.

C. PKC (Protein Kinase C) pathway and oxidative stress

Elevated intracellular glucose activates PKC through de novo synthesis of diacylglycerol (DAG). Activation of PKC in the glomeruli has been associated with processes increasing mesangial expansion, thickening basement membrane, endothelial dysfunction, smooth muscle cell contraction, and activation of cytokines and transforming growth factor-β (TGF-β). PKC induces oxidative stress by activating mitochondrial NADPH oxidase ***(Bedard et al., 2007).***

D. Hexosamine pathway

The hexosamine pathway stems from the third step of glycolysis, fructose-6-phosphate, which is converted to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). Glucosamine-6-

phosphate is then used as a substrate to increase transcription of inflammatory cytokines tumor necrosis factor-α (TNF-α) and transforming growth factor-α1 (TGF-α1) ***(Brownlee, 2001)***.

3. Inflammatory pathway:

Diabetic nephropathy is usually classified as a non-inflammatory glomerular disease; however, genome-wide transcriptome analysis studies consistently indicate the presence of inflammatory signaling pathways in the context of diabetic nephropathy ***(Woroniecka et al., 2011)***. Inflammation is considered to be one of the critical pathways that can limit regeneration in adult mammalian organs ***(Edinger and Thompson, 2004)***. The inflammatory response could be induced by multiple mechanisms, including hyperglycemia-induced cell death, which activates the influx of macrophages and other immune cells ***(Susztak et al., 2006)***. TNF-α signaling has also recently received significant attention because activation of this pathway predicted progressive diabetic nephropathy in a large patient population ***(Niewczas et al., 2012)***.

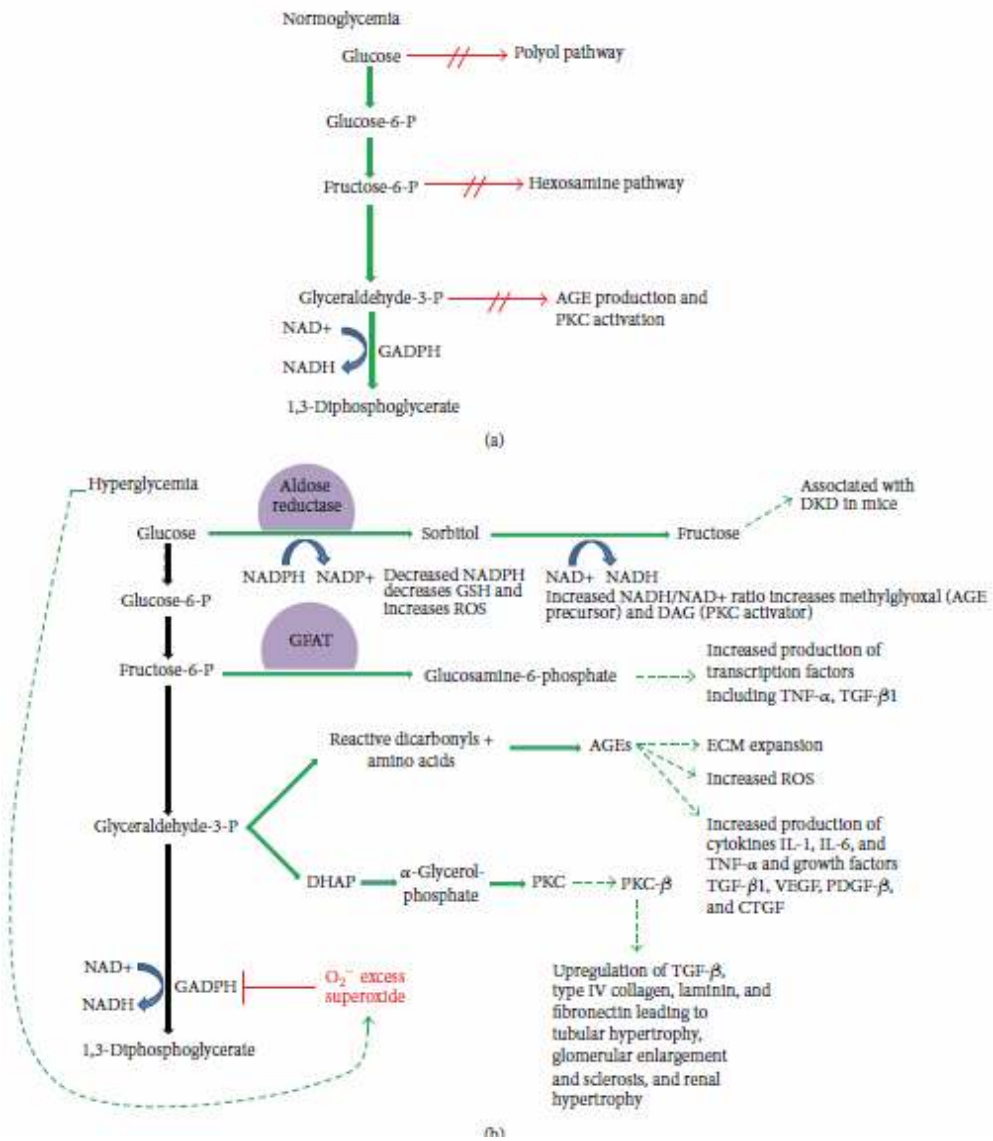

Figure (1): Summery of molecular pathways involved in diabetic nephropathy illustrating how hyperglycemia can result in kidney injury ***(Toth-Manikowski and Atta, 2015)***.

Assessment of renal function in diabetic nephropathy:

Staging kidney damage is accomplished by calculating the glomerular filtration rate (GFR). All diabetic patients should have a serum creatinine level on an annual basis. Once the creatinine level is obtained, the eGFR (estimated GFR) can be calculated with a formula provided by the National Kidney Foundation (NFK) ***(Shahady, 2014).***

Stage	Description	eGFR (mL/min/1.73 m^2)
1	Kidney damage with normal or increased GFR.	≥ 90
2	Kidney damage with mildly decreased GFR.	60-89
3	Moderately decreased GFR.	30-59
4	Severely decreased GFR.	15-29
5	Kidney failure	< 15

Figure (2): Staging of diabetic nephropathy on the basis of eGFR ***(Shahady et al., 2014).***

Biomarkers of diabetic nephropathy:

In the past, persistent microalbuminuria was the most studied biomarker in diabetic nephropathy. There are limitations in using albuminuria as a marker of DN as many patients experience GFR loss without deterioration in albuminuria and even normoalbuminuria ***(Perkins et al., 2010).*** In fact, histologically proven advanced diabetic glomerular

lesions can develop despite normoalbuminuria ***(Caramori et al., 2003).*** Furthermore, microalbuminuria is a lesser predictor of disease progression than macroalbuminuria ***(Perkins et al., 2007).*** Therefore, there is interest in finding biomarkers to detect DN earlier and identify progression risk.

Of the emerging candidate biomarkers, serum cystatin C, fibroblast growth factor 23 (FGF23) and soluble tumor necrosis factor (TNF) receptors have provided the most promising data ***(Krolewski et al., 2012).*** Another two potential novel biomarkers of progression in diabetic nephropathy have recently been identified: pigment epithelium-derived factor (PEDF) and fibroblast growth factor 21 (FGF21) ***(Lee et al., 2015)***. Also β2-Microglobulin shows a similar relationship with urine albumin in diabetic patients ***(Bellei et al., 2008).*** Novel biomarkers that may be useful in assessing early nephron injury in patients with diabetes includes Neutrophil gelatinase-assiociated lipocalin (NGAL) which showed correlation with the progression of albuminuria from absent to severe ***(Zachwieja et al., 2010),*** N-acetylglucosaminidase (NAG) which increases with the development and progression of microalbuminuria ***(Nauta et al., 2011),*** Kidney injury marker-1 (KIM-1) which was found to be correlated with regression of microalbuminuria ***(Vaidya et al., 2011)*** and π-Glutathione-S-Transferase (π-GST) that was found to increase across the spectrum of normo-, micro-, and macroalbuminuria ***(Cawood et al., 2010).*** There is also interest in urine microRNA profiling