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New Insights into the Interaction between Increased Iron-Deposition in the Kidney and Vitamin D/Klotho Axis in Diabetic Nephropathy

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Abstract

Diabetic Nephropathy (DN), a chronic complication of diabetes, is characterized by glomerular hyper-trophy, albuminuria, decreased glomerular filtration rate, and renal fibrosis resulting in end stage renal disease. Diabetic Nephropathy is one of the major micro-vascular complications of long term diabetes mellitus. The pathogenesis of diabetic nephropathy is multifactorial. For many years it was consensus among scientists that hyper filtration and activation of the renin angiotensin aldosterone system is enough to develop kidney injury (diabetic nephropathy). The goal of this review is to describe new pathways involved in the pathogenesis of diabetic nephropathy. Recent studies showed that new pathways are involved. One of these pathways is the increased production of free radicals via oxidative stress due to increased iron deposition in the lysosomes of the proximal convolute tubules. The increased oxidative stress in the lysosomes of the proximal convolute tubules can down regulate Klotho Protein expression and the synthesis of active vitamin D. The decrease in Klotho, active vitamin D and his receptor can aggravate the progression of diabetic nephropathy. AGEs-induced increased oxidative stress, also activated PKC-induced increased production of cytokines, chemokine's and different inflammatory and apoptotic signals.

Another pathway involved in the pathogenesis of diabetic nephropathy is the altered autophagy process via hyperglycaemia induced activation of the mTORC1 in this review we will concentrate on new data published recently on these pathways involved in the pathogenesis of diabetic nephropathy and new treatments proposed.

Keywords: Diabetes mellitus; Haptoglobin; Iron oxidative stress; Klotho vitamin D; Vitamin D receptor

Abbreviations

- DN=Diabetic Nephropathy
- RAASi=Renin-Angiotensin II-Aldosterone System inhibitors
- *Hp*=Haptoglobin
- PCT=Proximal Convolute Tubule
- SGL2i=Sodium-Glucose Transport Inhibitor

Introduction

Diabetic Nephropathy is one of the major micro-vascular complications of diabetes mellitus resulting in end-stage renal disease (ESRD) necessitating renal replacement therapy within 20 years of DM onset. More than 40% of patients with DM will develop diabetic nephropathy (DN) after 10 years of diabetes mellitus onset. The number of patients suffering from DM is increasing every year. The pathogenesis of DN is multifactorial including genetic and environmental factors [1,2]. Risk factors such as arterial hypertension and genetic factors are important in the development of DN.

Its morphologic characteristics include glomerular hypertrophy, basement membrane thickening, mesangial expansion, tubular membrane hypertrophy, tubulo-interstitial fibrosis and arteriolar thickening and finally global glomerulosclerosis. Micro-albuminuria is an early sign of glomerular damage. Different pathways are involved in the pathogenesis of DN, and involved altered intracellular metabolism associated with hyperglycemia (Glucose toxicity), including the activation of protein kinase C, renin-angiotensin-aldosterone axis (RAAS) and the accumulation of advanced glycation end-products, accelerated oxidative stress, and altered apoptosis (Figure 1).

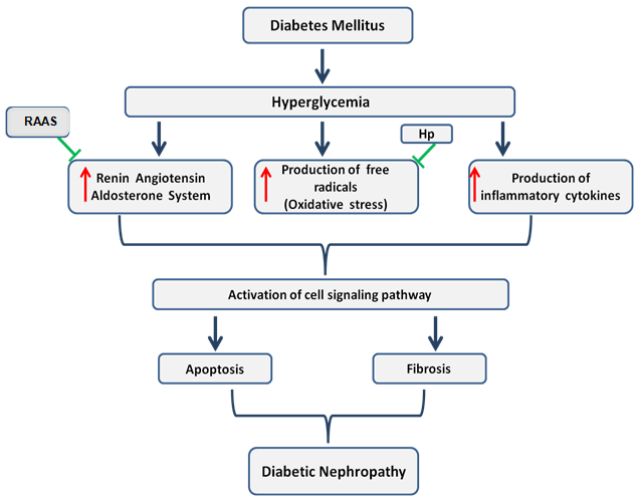

Figure 1: Schematic presentation of the different pathways involved in the pathogenesis of diabetic nephropathy and hyperglycemia. High glucose activate the renin angiotensin aldosterone system, increased free radicals production and inflammatory cytokines, with subsequent apoptosis and accelerated fibrosis. The prottective effect of the RAAS inhibitors on the development of diabetic nephropathy and the protective effects of *Hp 1-1*. Green Arrow=Inhibitory effects, Red Arrow=Stimulatory effects

Several lines of evidence have supported the concept that there exists polymorphic genetic loci which determine susceptibility to DN [3,4]. The importance of identifying a predictive marker would permit early identification of individuals at high risk of developing tubular and glomerular damage (Proximal and glomerular theory), in whom more aggressive blood glucose and blood pressure control might be initiated earlier, and may provide additional protection against the generation and progression of DN toward end stage renal disease. Understanding the new pathophysiology pathways of DN, and the development of new more specific therapies, such as new anti-diabetic drugs such as SGL2 inhibitor (SGL2i), and anti-oxidants such as vitamin E and iron chelating agent, can be of benefit in the future.

Early and chronic use of Angiotensin Converting Enzyme Inhibitors and Angiotensin Receptor Blockers or a combination of both in DM patients, have limited renal protection, and can explain the increasing number of DM patients reaching the dialysis units or kidney transplantation [5]. Recently different pathways were suggested as mediators for hyperglycemia causing renal tissue damage. Between these mediators are the Haptoglobin (*Hp*) gene polymorphism, free iron release, oxidative stress, active vitamin D (1,25D3), Vitamin D receptor (VDR) and Klotho gene [6,7]. The presence or activation of autophagy was found to play a protective role in human and mice podocytes against high glucose-induced cell injury, which indicates a novel cellular mechanism and provides a potential therapeutic target for diabetic nephropathy, such as Metformine and SGL2i.

The Different Pathways

Haptoglobin (*Hp*)

Increased oxidative stress with free radicals, has been proposed to play a fundamental role in the development of DN [8,9]. Therefore, genetic loci encoding proteins regulating the level of oxidative stress are potential candidate susceptibility genes for DN. Different candidate genes have been identified in genetic association studies with DN [10]. One of these genes was *Hp*, a serum antioxidant protein which serves to protect against oxidative stress induced by extracorpuscular hemoglobin and iron release [11]. As reactive oxygen species, particularly those derived from iron, have been implicated in the progression of DN and other vascular complications of Diabetes, polymorphic genetic loci encoding variants in enzymes protecting against iron-induced oxidative stress serve as potential susceptibility determinants for the development of DN.

There are two common alleles at the *Hp* locus, denoted 1 and 2. The structure and function of the two *Hp* allele protein products are distinct. We and others have shown in vitro and in vivo that the *Hp 1* protein is a superior antioxidant to the *Hp 2* protein [12,13].

The two *Hp* alleles are in a balanced polymorphism with 40% of the alleles being *Hp 1* and 60% *Hp 2*. In Israel the distribution of *Hp 1-1* is (16%), *Hp 2-1* (48%), and *Hp 2-2* (36%) [14], in diabetic and non-diabetic individuals. We examined the association of DN and the *Hp* polymorphism and found that the *Hp 1-1* genotype was associated with a significantly lower prevalence of DN [15,16] and an apparent slower rate of progression of DN to ESRD [17,18] compared with individuals with *Hp 2-1* and *Hp 2-2*.

The *Hp 2* allele is found only in humans [19]. All other animals (mice) have only the *Hp 1* allele and therefore the *Hp 1-1* genotype. One approach to model the *Hp* polymorphism in mice is to introduce the human *Hp 2* allele as a transgene by producing a transgenic mouse with a genetically engineered murine *Hp 2* allele and targeted insertion of this murine *Hp 2* gene to the murine *Hp* locus by homologous recombination [20-22].

To study the importance of *Hp* genotype on the incidence and progression of diabetic nephropathy, we used diabetic mice with different *Hp* genotype (*Hp 1-1*, *Hp 2-2*, *Hp* 2-1). These mice become type 1 diabetes mellitus (DM) by using intraperitoneal Streptozotocin (STZ) administration at 6 weeks of age in a low-dose 5-day protocol, approved by the NIH (50 mg/kg for 5 days), were used to study the mechanisms of the different proposed pathways. We published different studies in human and mice showing the better protective effects of *Hp 1-1* genotype against the development of vascular complications of DM.

Iron and oxidative stress

Iron plays an important role in maintaining physiological homeostasis in the body. However, excess iron can lead to free radical damage via the Fenton reaction, resulting in cell damage.

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Reactive oxygen species (ROS), particularly those derived from excessive labile iron, have been implicated in the increase of oxidative stress injury in the renal proximal convolute tubular cells (PCT), and podocytes with progression of DN and other vascular complications of DM [23,24]. The persistent state of hyperglycemia affects the renin-angiotensin system and the signaling of the transforming factor (TGF-β) with consequent chronic inflammation and sclerosis.

The major function of the *Hp* protein is to bind and modulate the fate of extra-corpuscular hemoglobin and its iron load. This complex of *Hb-Hp* is cleared from the circulation by the receptor CD 163 on the macrophage. It is important to mention that the efficiency of the *Hp* 1-1 to clear the free iron from the circulation via the CD 163 receptor, is more efficient than *Hp-2-2*.

We have previously demonstrated an interaction between the *Hp* genotype and DM on the increased accumulation of iron in the lysosomes of the renal PCT cells [25,26]. As we mentioned before, in *Hp 2-2* there is massive glomerular filtration of iron in the capillary wall, with increased reabsorption and lysosomal deposition, in the PCT cells.

We used special transmission electron microscopy, (TEM) and and electron energy loss spectroscopy (EELS) to demonstrated the marked accumulation of electron-dense deposits in the lysosomes of proximal tubules cells in *Hp 2-2* DM mice [25]. These deposits were iron rich, and are associated with lysosomal membrane lipid peroxidation and loss of lysosomal membrane integrity with consequent decrease in different proteins expression. Klotho and vitamin D and his receptor are part of these proteins.

Kan Saito et al. have shown that iron overload decreased renal expression of the klotho gene at both the mRNA and the protein level, and that iron chelation can upregulate klotho expression [27]. Their findings suggest that, the mechanisms of downregulation of klotho may be mediated by abnormal iron metabolism in the kidney elicited by angiotensin II [28].

Iron-induced renal tubular injury via oxidative stress and apoptosis, and altered klotho level, may play a major role in the development of DN, and may be a future therapeutic target such as with chelating agents such as Deferiprone and vitamin E, for slowing the progression of DN [29].

Klotho is a novel anti-aging gene encoding a protein with a multiple pleiotropic effects. αKlotho gene is composed of five exons, in humans and mice, it's highly expressed in the distal and proximal convolute tubular epithelium of normal kidneys. The soluble α klotho is characterized by his anti-apoptotic effects especially in DN. Several studies have shown that α klotho expression is decraesed in the renal PCT cells in early stages of DN, in humans and mice model [30,31]

Interesting cross talk exist between α klotho-Vitamin D/ Vitamin D receptor- (VDR) axis. Up regulation or restoration of klotho by 1,25(OH)2D3, may provide a mean to slow down the progression of chronic diabetic kidney disease (CDKD). Klotho slows the renal deterioration and the cardio-vascular complications via different inhibitory effects on Transforming Growth Factor β (TGF-β), and suppressive effects on reactive oxygen species production (ROS). klotho also exerts inhibitory action on renal fibrosis and cardio-vascular atherosclerosis in high oxidative stress conditions such as hyperglycemia and DM (Figure 2) [30-35]. Our unpublished results show that diabetic *Hp 2-2* mice and humans have reduced expression of klotho in the PCT cells in early and late stages of DM vs *Hp-1-1*.

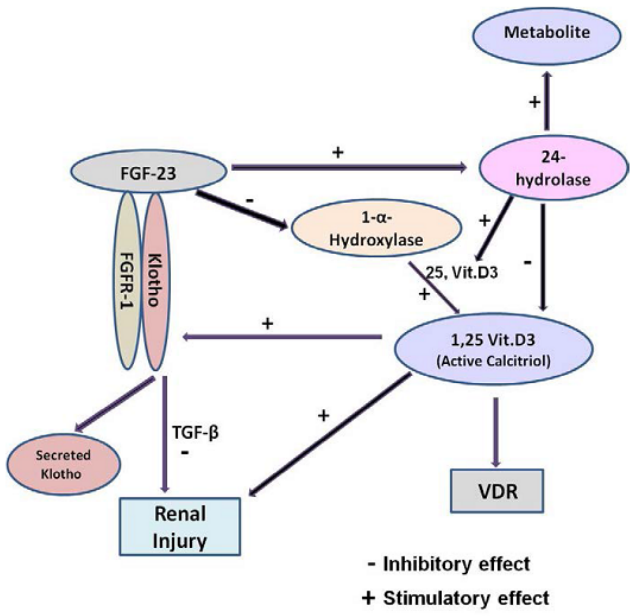

Figure 2: Schematic illustration of the klotho–vitamin D axis cross talk. Normal expression of renal Klotho is required for normal vitamin D homeostasis via down-regulation of 1-α-hydroxylase transcripts and upregulation of 24-hydroxylase. Likewise calcitriol stimulates both Klotho and FGF-23, and both FGF-23 and Klotho inhibits calcitriol. The podocytes express the VDR that is highly inducible by 1,25(OH)2D3, and, through VDR, 1,25(OH)2D3 transcriptionally stimulates the expression of nephrin, a key slit diaphragm protein synthesized by podocytes.

Vitamin D/VDR signaling

Vitamin D is biologically inactive and requires enzymatic conversion to active metabolites. Vitamin D is transported to the liver, where it is first hydroxylated to yield 25-hydroxyvitamin D. Then 25-Hydroxyvitamin D is further hydroxylated by 1-α-hydroxylase in the PCT of the kidney, to the active form 1,25-dihyroxyvitamin D [36-38]. The active 1,25-dihydroxyvitamin D binds to the intracellular vitamin D receptor (VDR) to activate vitamin D response elements within target genes [39]. In the kidney, vitamin D is important for maintaining podocytes integrity, and suppressing renin gene expression and inflammation [39,40]. 1,25- dihydroxyvitamin D progressively decrease due to PCT injury by iron and ROS production, leading to a vitamin D deficient state. The Vitamin D analogues supplementation as Calcitriol or Paricalcitol (19-nor-1,25-dihydroxyvitamin D2), improves the renal damage of diabetic kidney disease patients and their survival (Figure 2) [41,42].

Increasing prevalence of DN and ESRD in spite of massive RAAS inhibition (ACEI/ARBs), has made the need for new effective treatment of DN. Thereby identifying new therapeutic targets beyond RAAS inhibitors is required in order to improve clinical management. Recently Wang et al. from Chicago [41] showed that vitamin D/VDR signaling in podocytes plays a critical role in the kidney protection from diabetic injury and reconstitution of VDR null mice with the human VDR (hVDR) transgene in podocytes rescued the severe diabetes-related renal damage. Experimental studies showed that administration of a selective vitamin D analogue like Paricalcitol reduces albuminuria [42].

Furthermore, recent data suggest that Paricalcitol, added to renin angiotensin aldosterone system (RAAS) blockade, further reduces albuminuria in people with Type 2 diabetes and diabetic nephropathy. The Renin inhibitor (Aliskiren) plus Paricalcitol, significantly decreased interstitial fibrosis volume when compared to monotherapy [43,44].

Autophagy

Autopahagy is a highly regulated lysosomal protein degradation pathway that removes protein aggregates and damaged or excess organelles in order to maintain intracellular homeostasis and cell integrity. Recently a review by Mary Choi and her group [42] showed that the accumulation of damaged proteins and organelles is associated with the pathogenesis of DN. Autophagy pathway in the kidney is activated under high glucose conditions, with increased oxidative stress via free radicals in PCT cells, and in podocytes. The formation of autophagosomes depends on several genes including Beclin-1, and other autophagy-related (*Atg*) genes, especially *Atg-5*. Genetic evidence have led to the hypothesis that autophagy is involved in the pathogenesis and progression of DN (i.e., polymorphisms in *ATGs*).

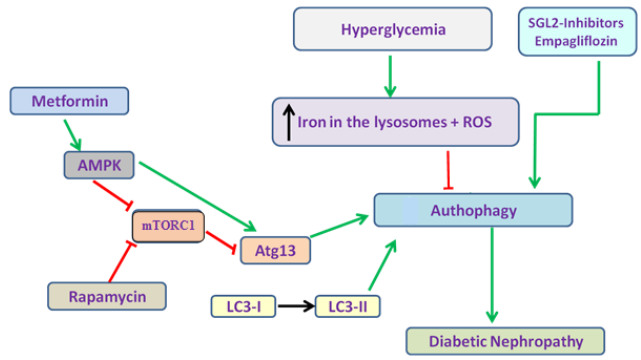

Figure 3: Schematic presentation of autophagy process down regulation in condition of high glucose via increased production of free radicals. The up regulation of the autopahgy process with the new anti-diabetic drug SGL2i, and the mechanism of action of the old drug Metformin via blocking mTORC1 and *atg13.* Green Arrow=Stimulatory effects, Red Arrows=Inhibitory effects.

The autophagy-lysosomal degradation pathway is likely to play an essential role in maintaining podocyte function and integrity. Podocytes exhibit active autophagy process under normal glucose levels, suggesting that podocytes require a high basal level of autophagy to maintain podocytes and glomerular maintenance [42-45]. Furthermore, it is interesting that the loss of autophagy in podocytes affects the ultrastructure and function of these cells but also that of nearby mesangial cells, which become sclerotic.

New evidence suggests that targeting the autophagic pathway to activate and restore autophagy activity may be Reno-protective in diabetic patients, especially via the mTORC1 (Figure 3) [45].

Discussion

In humans, hyperglycemia has been shown to be necessary, but not sufficient, to induce the development of micro- and macrovascular complications. Genetic differences between diabetic patients are important in the determination of why some patients develop these complications and others do not. To investigate and treat diabetic patients in early reversible stages, we used our diabetic mice model. As we have shown in an earlier publication [26], the typical structural changes of DN, were increased iron deposition in the lysosome of the renal PCT cells, which can increase the generation of reactive oxygen species and cell damage [26].

Hp 2-2 DM in humans and mice has more renal damage compared with the protective *Hp 1-1* DM [16]. This can be explained by differences in the manner in which the two *Hp* types regulate the disposition of extra corpuscular hemoglobin and more specifically hemoglobin-derived iron [3,4]. *Hp 1-1* protein is superior to the *Hp 2-2* protein in binding to this free hemoglobin and neutralizing its oxidative potential. Furthermore, the rate of clearance of hemoglobin is *Hp* type dependent. *Hp 1-1* directs a more rapid clearance of free hemoglobin via uptake by the CD163 macrophage scavenger receptor. In the diabetic state, this is particularly important as the ability of *Hp* to block the oxidative activity of hemoglobin is impaired when hemoglobin becomes glycated [12,13]. Patients and mice with the *Hp 2-2* genotype, the clearance of iron is impaired and are prone to microvascular complications (Nephropathy and Retinopathy) with increased mortality [13-15]. Iron chelation by oral Ferriprox (Deferiprone) has a renoprotective effect in DN rats by relieving oxidative stress, inflammation, and fibrosis. Further, hyperglycemia increases both iron accumulation and cell senescence in PCT cells, and macrophage infiltration in the kidney of STZ-induced type 1 diabetic mice. The inhibition of dietary iron absorption by Desferroxamine (DFX) suppressed the increase in proximal tubular iron accumulation and macrophage infiltration into the interstitial space.

Due to the increase in number of patients with type 2 DM with diabetic nephropathy reaching the hemodialysis unit, in spite of chronic and maximal dose of ACEI and ARBs, blocking the RAAS, other agents such as anti-oxidant and iron chelators were investigated. Between these is vitamin E supplementation

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to DM type 2 patients. Our results had demonstrated that vitamin E appears to provide renal protection to *Hp 2-2* DM mice but does not appear to have any effect on *Hp 1-1* DM mice [45,46]. The pharmacogenomic implications of these findings are significant. Clinical Studies assessing the effect of vitamin E on the progression of DN in humans with DM are not conclusive. Moreover, recent meta-analysis suggested that there is an increased risk of all cause mortality with high-dose vitamin E supplementation. The ability of vitamin E to reduce features of renal disease characteristic of early human DN in *Hp 2-2* DM mice but not in *Hp* 1-1 DM mice suggests that there may also be an interaction between *Hp* genotype and vitamin E therapy on diabetic renal disease [25,26]. We had published that the concentration of vitamin E in the lysosomes purified from *Hp 2-2* DM mice kidneys was significantly lower than in lysosomes of *Hp 1-1* [25].

Currently we are studying the consequences of increased iron deposition in our mice model with *Hp 2-2* on the vitamin D-vitamin D Receptor-klotho axis. Our preliminary results showed a decrease in the α-1 hydroxylase, vitamin D receptor expression and increased apoptosis. All these alterations can be mediators in the development and progression of DN [1,2].

It is generally accepted that klotho expression in the kidney is markedly decreased in the early stages of DN in mice and humans [47-49]. Osamu Asai and his group [49], had published a paper on the role of klotho in special DN mice. In this study, they showed that renal Klotho expression levels were decreased in patients with early DN and in the streptozotocin (STZ) induced mouse model of T1D. Other study by Lee and his group [50] were the first to demonstrate that plasma and urine levels of soluble Klotho are significantly elevated in the DM patients compared to control subjects. Hence, klotho expression levels were decreased in kidneys of patients with early DN but with increased levels of plasma and urinary klotho. Although the exact mechanism of how klotho is reduced in diabetic kidney disease is not well understood, but the increased oxidative stress, and Ag-II may be involved [27,28]. As we mentioned before the increased iron deposition in the lysosomes of PCT cells, via Ag-II, can down regulate Klotho expression in the PCT. Accordingly, soluble Klotho may serve as a biomarker as well as a pathogenic factor for the progression of DN [28].

The role of active Vitamin D in the treatment of early stages of DN is not well understood or proven. The activities of 1,25 (OH)2D3 are mediated by the VDR, a member of the nuclear receptor super family. The so-called non-calcemic activities include regulation of renal and cardiovascular functions [36,40]. Relevant examples of these non-calcemic activities are the regulation of the RAAS in the kidney [38,39]. This includes the maintenance of podocytes integrity, suppressing renin gene expression and inflammation. Therefore, vitamin D has the potential to have a favorable impact in DN via VDR in kidney protection from DM oxidative stress injury [40,41]. Li and his group from Chicago [51] showed that Paricalcitol, the selective vitamin D receptor, has a podocyte protection and integrity in DN mice. The synergistic therapeutic effects of combined vitamin D analog Paricalcitol (19-nor-1,25-dihydroxyvitamin D2) with AT1 receptor antagonist (Losartan) on kidney disease in a model of type 2 diabetic mice, showed a dramatic therapeutic synergism, manifested by prevention of progressive albuminuria, restoration of the glomerular filtration barrier, reversal of the decline in slit diaphragm proteins, and reduction of glomerulosclerosis [43,52]. The expression of VDR in kidney tissue was significantly decreased in early stages of DN, and the down-regulation of VDR could be restored to the normal level by the treatment with Paricalcitol. Furthermore, the beneficial effects of Paricalcitol on renal fibrosis in DN is mediated by VDR via restoration of klotho expression [53]. Other studies had shown contoversial results on the effect of vitamin D on the kidney and heart. A review from Italy by Ciccone et al, discuss that hypovitaminosis D is associated with increased mortality from stroke and heart failure, and that administration of therapeutic vitamin D can increase mortality. Their conclusion was that the the role of vitamin D supplements in improving cardiovascular outcome in patients with diabetes with hypovitaminosis D remains to be determined [54].

Several studies published recently on autophagy as a new pathway involved in the pathogenesis of DN. The autophagy system is more than housekeeper system. The autophagy process is altered in both podocytes and PCT cells under high glucose condition and DM [55]. Autophagy can be induced by high glucose levels in various cell types including the podocytes, partly through hyperglycemia-mediated production of reactive oxygen species, and it has protective effects in vitro. In podocytes, high glucose levels lead to podocyte apoptosis in vitro that is mediated through caspase-3 activation.

Interestingly, in podocytes of diabetic mice and patients, mTORC1 is highly activated and may be involved in the mechanisms of diabetes-related autophagy inhibition in podocytes. These results suggest that the mTORC1-autophagy axis may be a future therapeutic target in diabetic nephropathy. The therapeutic potential of autophagy in DN, by the regulators of autophagy, especially via the mammalian target of Rapamycin (mTOR) complex 1 (mTORC1) [56]. Rapamycin, a potent mTORC1 inhibitor, can ameliorate glomerular lesions in diabetic animal models.

The acute exposure to high glucose-induced autophagy, which is mediated through the ANG-II with increased generation of ROS in podocytes. Recent papers published had shown that new anti-diabetic drugs such as, Dapagliflozin and others contain a potent selective reversible inhibitor of SGLT2, expressed exclusively in the proximal tubule of the kidney [57-59]. Between the new anti-diabetic medications that target a specific pathophysiologic pathway, the SGLT2 inhibitors and Metformin are of major interest in the future, in the treatment of early stage DN. Since SGLT2 inhibitors act via a different mechanism of action than metformin, the combination therapy of dapagliflozin and Metformin, is beneficial to treat T2DM and DN [59].

Conclusion

lysosomal iron overload decreased renal expression of klotho at both the mRNA and protein level, and that low iron diet and iron chelation drugs suppressed the angiotensin II-induced down regulation of this gene [59,60]. Furthermore, a free radical

scavenger also suppressed the angiotensin II-induced downregulation of klotho, supporting the involvement of an increased production of reactive oxygen species in this process. This cross-talk between the free iron and klotho-Vitamin D-VDR axis is of great importance in understanding and treating our DN patients in the future.

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