AN EBOOK ON

TYPE 2 DIABETES

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An eBook on Type 2 Diabetes

Chapter 1

ROS acts as a double-edged sword in the pathogenesis of Type 2 Diabetes Mellitus

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Abstract

Although the clear mechanism of T2DM is still to be elucidated, it has been well established that reactive oxygen species (ROS) derived from multiple sources plays a causal role in multiple types of insulin resistance and contributes to β-cell dysfunction thus enhances the development and progression of T2DM. What is incomprehensible is that the detrimental ROS also plays a substantial role in the normal insulin signal transduction and glucose-stimulated insulin secretion (GSIS) in β-cell, which forces us to re-recognize the role of ROS under physiological and pathological conditions in a more broad way. Redox homeostasis is tightly controlled by the transcriptional factor nuclear factor erythroid 2-related factor 2 (Nrf2), whose abnormality is believed to be related with diabetes. Accumulating evidences suggest that there are important cross-talks between Nrf2 and PPARγ, PGC1α, PI3K/Akt on regulating antioxidant enzymes and the development of diabetes. Therefore, these evidences indicate that Nrf2 may be a critical element in taking survival and death decisions when cells are exposed to an oxidant environment. In conclusion, enhancing GSIS and insulin sensitivity through the regulation of Nrf2 levels is a potential avenue for developing new therapeutics. Nrf2 may become a promising target for the treatment of T2DM.

Keywords: Reactive oxygen species; Type 2 diabetes mellitus; Nrf2; Antioxidant; MAP kinase; PI3K/Akt

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1. The prevalence of type 2 diabetes mellitus

Estimates by the American Diabetes Association (ADA) indicate that over the past three decades, there has been an explosive increase in the prevalence of diabetes mellitus (DM) [1, 2] , and current estimates suggest that by the year 2030, over 350 million people worldwide will be afflicted with this disease and its debilitating conditions [3] , leading to a series of complications such as cardiovascular disease, nephropathy, retinopathy and widespread disease of both the peripheral and central nervous systems. Type 2 diabetes mellitus (T2DM) represents at least 80-85 percent of all DM and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index [4]. The incidence of undiagnosed diabetes, impaired glucose tolerance and impaired fasting glucose levels raises future concerns in regards to the financial and patient care resources that will be necessary to care for patients with T2DM. Therefore, it is very important to examine the molecular mechanism of T2DM and to explore a therapeutic target for T2DM.

2. Pathogenesis Of T2DM: Insulin Resistance and B-Cell Dysfunction

T2DM is a heterogeneous disorder characterized by hyperglycemia resulting from defects in insulin sensitivity (insulin resistance) and/or secretion (pancreatic β-cell dysfunction). Under normal condition, insulin lowers the level of blood glucose through suppression of hepatic glucose production and stimulation of peripheral glucose uptake, in which process any dysfunction can lead to insulin resistance. Normal β-cells can compensate for insulin resistance by increasing insulin secretion or β-cell mass, leading to more insulin in circulation, but insufficient compensation leads to the onset of glucose intolerance, resulting in the aggravation of hyperglycemia. Although the clear mechanism of T2DM is still to be elucidated, it has been well established that hyperglycemia-induced generation of reactive oxygen species (ROS) contributes to the development and progression of T2DM and its related contributions [5, 6]. Therapies attributing to lessen oxidative stress could contribute to improve glycemia control and prevent complications of T2DM [7,8]. Moreover, a number of studies have implicated that excessive ROS plays a pivotal role in both β-cell dysfunction [9] and multiple types of insulin resistance [10].

3. Paradoxical roles of ROS in the pathogenesis of T2DM

3.1. Oxidative stress: imbalance of the generation and elimination of ROS

Systemic oxidative stress is defined as an imbalance between the generation of ROS (also known as free radicals, including superoxide (O_2•−), hydrogen peroxide (H_2O_2), peroxynitrite (•ONOO−), and hydroxyl (•OH−)) and the body's ability to eliminate these species via endogenous antioxidant defenses [11-14], including phase II detoxifying and antioxidant enzymes, as well as non-enzymatic scavengers of ROS and metal ions [15]. Under physi-

ological conditions, the antioxidant defenses are able to protect against the deleterious effects of ROS, but under conditions when either the ROS generation is increased or the antioxidant defenses are decreased, ROS accumulates, leading to cellular and tissue damages [16]. Indeed, ROS acts directly through oxidative damage on macromolecules (proteins, lipids, DNA) or indirectly through activating a number of signal transduction pathways sensible to stress mechanisms, finally resulting in oxidative stress and cell dysfunction.

3.2. Oxidative stress and insulin resistance

Under normal conditions, insulin binds to its receptor (insulin receptor, IR) on cell surface, and then IR and insulin receptor substrate (IRS) will be phosphorylated, which results in activation of various insulin signaling pathways, leading to glucose transportation, glycogen synthesis and protein synthesis (Fig.1). Under insulin resistant conditions, main insulin target tissues, including liver, muscle and adipose, become insensible to insulin. A complex network of insulin signaling pathways is involved in the patho-physiology of insulin resistance. Oxidative stress is believed to modify a number of the signaling pathways within a cell that can ultimately lead to insulin resistance. Previous studies had shown that the inhibitory effect of ROS on insulin/ insulin-like growth factor 1 (IGF-1) receptor signaling is mediated through activation of serine/threonine kinases [17], which in turn phosphorylate IRS-1 and IRS-2 and cause insulin/IGF-1 resistance, since ser/thr-phosphorylated IRS proteins dissociate from the insulin receptor. As a result, IRS-1 and IRS-2 can no longer activate phosphatidylinositol 3-kinase (PI3K) and thus impair insulin-induced glucose transporter type 4 (GLUT4) translocation and glucose transportation [18]. It is believed that mulitiple so-called stress kinases, such as c-Jun N terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), nuclear factor κB (NFκB) and certain protein kinase C (PKC) isoforms, are involved in the desensitization of insulin (Fig.1). The common characteristic of these kinases seems to be their ability to directly or indirectly increase serine/threonine phosphorylation of IRS proteins induced by ROS, thus attenuating normal insulin signaling (Fig.1) and resulting in insulin/IGF resistance due to dissociation of IRS from the receptor and PI3K downstream signaling [19]. These results are consistent with the data obtained with adipocytes, which demonstrates a causal role of ROS in insulin resistance [20]. Taken together, these data indicate that excessive generation of ROS has a causal role in multiple forms of insulin resistance [20].

3.3 Oxidative stress and pancreatic β-cell dysfunction

Disruption of insulin synthesis and secretion are mainly considered to be due to chronic hyperglycemia in T2DM. Once hyperglycemia becomes apparent, β-cell function gradually deteriorates. Chronic oxidative stress is an important cause of glucose toxicity in β-cells in T2DM [21]. Compared with other tissues, the expression of antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (Gpx), are very low in β-cells [22,23], thus β-cells

are thought to be a target of oxidative stress-mediated tissue damage [23]. So, it is probable that oxidative stress is an important event involved in β-cell deterioration in T2DM. It has been previously shown that oxidative stress suppresses the insulin gene transcription in β-cells [24] . Pancreatic homeobox factor-1(Pdx-1) and MafA are two important transcription factors for normal insulin gene expression. Antioxidant treatment of β-cell lines and models of T2DM were shown to protect against deterioration of insulin gene expression induced by exposure to high glucose, indicating that oxidative stress was responsible for the decrease of Pdx-1 and MafA [25]. Taken together, these results suggest that ROS is a central influencing factor that contributes to insulin resistance and β-cell dysfunction in T2DM and its complications (Fig. 2).

3.4. ROS also acts as a beneficial signal in the production of insulin and insulin action

Paradoxically, certain level of ROS may have insulin-mimicking effects, and for example, H_2O_2 can increase glucose transportation and inhibit lipolysis [26-28]. Moreover, Insulin stimulates the rapid production of H_2O_2 and this insulin-dependent H_2O_2 generation is not only involved in the regulation of tyrosine phosphorylation events in the early insulin signaling cascade but also has important effects on the regulation of downstream insulin signaling, including the activation of PI3K, Akt, and ultimately cellular glucose transport in response to insulin [29]. Recently, Loh et al reported that mice lacking one of the key enzymes responsible for the elimination of ROS, glutathione peroxidase 1 (Gpx1), were protected from high-fat-diet-induced insulin resistance [30]. Insulin sensitivity in Gpx1-/- mice was increased, attributing to the enhancement of insulin-induced PI3K/ protein kinase B (Akt) signaling and glucose uptake in muscle, which could be reversed by the antioxidant N-acetylcysteine [30]. Increased insulin signaling correlated with enhanced oxidation of the PTP family member PTEN, which terminates signals generated by PI3K [30]. These studies provide causal evidence for the enhancement of insulin signaling by ROS in vivo. Moreover, our recent studies (not shown) have also showed that the effect of ROS on insulin-induced phosphorylation of Akt was concentration-dependent. Relative low level of ROS, including H_2O_2, tBHP and glucose plus glucose oxidase, promoted the phosphorylation of Akt induced by insulin, but high level of ROS inhibited this insulin signaling, which was closely related with glucose metabolism and thus the pathogenesis of insulin resistance.

Defection of glucose stimulated insulin secretion (GSIS) in pancreatic β-cell is instrumental in the progression to hyperglycemia [31]. ROS generation occurs in glucose metabolism and correlates with insulin secretion. In recent years, more and more attention has been paid to the potential role of ROS in glucose signaling and insulin GSIS. The promoting effect of ROS on GSIS has been reviewed in detail by Pi et al [32], which suggests that ROS derived from glucose metabolism are potential metabolic signals that facilitate insulin secretion in β-cell. In conclusion, ROS may be not only the byproduct of metabolism or detrimental agent

but also a potential signal molecular in insulin signaling and GSIS in β-cells (Fig. 3). Indeed, ROS acts as a double-edged sword under normal physiological and pathological conditions.

4. Mitochondria, ROS and T2DM

Mitochondria function as the "power house" in a cell to produce adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. During the process of ATP production, electrochemical proton gradient across the mitochondrial inner membrane is required. There are five enzyme complexes that are important for the transportation of electrons, including Complex I (NADH coenzyme Q reductase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc1 complex), Complex IV (cytochrome c oxidase), and Complex V (F0F1 ATP synthase).

Electron transport chain (ETC) creates an electrochemical proton gradient through the regulation of redox reaction. It is considered that 85-90% of the O_2 in a cell is consumed in mitochondrial ETC and approximately 1-3% of those O_2 is incompletely metabolized and diverted into O_2•- in complex I and complex III. The production of ROS in mitochondrial ETC is obligatory and the amount of ROS generation varies with the redox state of the electron carriers under certain conditions of the mitochondrial oxidative metabolism [29,30]. Moreover, recent evidence has shown that several other enzymes in mitochondria, such as P66Shc, a main isoform of SHC-transforming protein 1 (SHC1), cytochrome p450 system and uncoupling proteins (UCPs) could also contribute to ROS generation.

Mitochondria are the most important source of ROS in most of the mammalian cells. Mitochondrial ROS generation in the OXPHOS process contributes to mitochondrial dysfunction through interaction with mitochondrial and cellular components such as DNA, proteins, lipids, and other molecules. Mitochondrial dysfunction is a state that mitochondrial biogenesis is diminished, membrane potential is altered, mitochondrial number is decreased and the activities of oxidative proteins are altered. It is reported that mitochondrial ROS acts as either essential physiological signals in several physiological processes, such as insulin signaling transduction and GSIS, or major causes of numerous diseases, including diabetes, neurodegenerative diseases, cancer, immune, cardiovascular, kidney, and hepatic diseases, and aging etc. Mitochondria-generated ROS and mitochondrial dysfunction are closely involved in insulin resistance, β cell dysfunction and hyperglycemia. However, it is still not clear whether insulin resistance is the primary cause of mitochondrial dysfunction or vice-versa. Targeting mitochondria might be a promising strategy for potential therapeutic purposes in metabolic disorders.

5. Regulation of redox balance by Nrf2

To avoid damage by ROS, a tight regulation of the cellular redox balance is required,

which is achieved at least in part by the action of nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 is a member of the "cap 'n' collar" basic leucine zipper family of transcription factors (CNC-bZIP) that can transactivate antioxidant responsive element (ARE) [33-35]. Nrf2 is broadly expressed in tissues, which is kept in the cytosol by kelch-like ECH-associated protein 1 (Keap1) under non-stressful conditions and undergoes proteosomal degradation through a specific ubiquitin–26S proteasome pathway by the Keap1/Cul3-dependent ubiquitin ligase (E3) [36]. Interaction between the inducers and the Keap1 protein through chemical–protein thiol interactions or Nrf2 itself through phosphorylation by kinase initiates the signaling transduction, leading to the stabilization and activation of the Nrf2 protein [37]. Upon recognition of signals imparted by oxidative and electrophilic molecules including ROS, including a number of antioxidants, heavy metals and certain disease processes [38], Nrf2 is released from Keap1, escapes proteosomal degradation and translocates to the nucleus to induce the expression of a series of genes involved in defense and survival [36]. Our and others' experiments [39,40] have shown that upon activation, Nrf2 mediates the induction of a spectrum of cytoprotective proteins including phase II enzymes and antioxidant proteins (Fig.4), including NAD(P)H: quinone oxidoreductase (NQO1), glutamate-cysteine ligase catalytic and modulatory subunits (GCLC and GCLM), peroxiredoxin (PRX) families, thioredoxin (TRX), glutathione S-transferases (GST), cytosolic copper-zinc superoxide dismutase (CuZnSOD), mitochondrial manganese superoxide dismutase (MnSOD) [41], Gpx, glutathione reductase (GR), heme oxygenase-1 (HO-1), through the ARE-dependent pathway [42-49].

6. The role of Nrf2 in the pathogenesis of T2DM

For the past few years, accumulative evidence demonstrates that Nrf2 plays a great role in the protection against diabetes. Glucose promotes ROS production in cardiomyocytes and Nrf2 is required for the control of ROS production in the cells under normal conditions and for high glucose-stimulated oxidative stress [50]. Hyperglycemia-induced oxidative stress has been reported to contribute to the dysfunction of renal mesangial or endothelial cells. The glomeruli of human diabetic nephropathy patients are under oxidative stress and Nrf2 levels are elevated [51]. In animal diabetic model, Nrf2 has been demonstrated to be crucial in ameliorating streptozotocin (STZ)-induced renal damage, indicating that Nrf2 plays a protective role in diabetic nephropathy, and that dietary or therapeutic activation of Nrf2 could be used as a strategy to prevent or slow down the progression of diabetic nephropathy [51]. Compared to wild-types, mice lacking Nrf2 (Nrf2-null) have lower basal serum insulin and prolonged hyperglycemia in response to glucose challenge. The absence of Nrf2 worsens hyperglycemia in type 1 diabetic mice, implicating that Nrf2 participates in glucose homeostasis [52]. In diabetic Nrf2-/- mice induced by STZ, hyperglycemia increased oxidative and nitrosative stress and accelerated renal injury [51, 53]. Moreover, Cheng et al [54] have reviewed the role of Nrf2-Keap1 defense pathway in endothelial cells in diabetes.

High levels of glucose accelerate the formation of advanced glycation end-products (AGEs). In turn, AGEs generate ROS and activate inflammatory signaling cascades through binding with the AGE-specific receptor (RAGE) [55] . AGE-modified bovine albumin (AGE-BSA) has been reported to induce ROS generation, leading to nuclear translocation of Nrf2 and Nrf2-dependent induction of the antioxidant genes, including HO-1 and NQO1, in bovine aortic endothelial cells [56] . And AGE-induced up-regulation of Nrf2-linked antioxidant enzyme activity was considered to play a protective role against sustained oxidative stress in diabetes [56].

Insulin-induced HO-1 mRNA and protein expression were observed in renal adenocarcinoma cells and mouse primary tubular epithelial cells [57]. The transcription factor Nrf2 was found to translocate to the nucleus following insulin treatment in a PI3K-dependent manner, which was responsible for the induction of HO-1 [57]. In addition, insulin-induced activation of PI3K and Nrf2 was also reported to protect against oxidative stress through up-regulation of GCLC in endothelial cells [58,59]. Decreased GCLC expression due to hyperglycemia and insulin deficiency can lead to decreased GSH levels that impairs activation of Nrf2 and antioxidant defense which indicates an important action of insulin is to increase the expression of GCLC through the activation of Nrf2 [60]. With regard to insulin's ability to induce GCLC expression, PI3K/Akt/mTOR signaling and consequent translocation of Nrf2 to nuclear was required [61]. The result was coinciding with the report which showed that chronic hyperglycemia resulted in enhanced apoptosis in human brain endothelial cells, which was attenuated by insulin [62].

A defect in insulin/IGF-1 signaling in the regenerating liver was identified in the Nrf2-deficient liver [63]. Consistent with data obtained with adipocytes [20], the chronic oxidative stress in hepatocytes of Nrf2-deficient mice resulted in resistance to exogenous insulin or IGF-1 in vitro. Alterations in the phosphorylation status of IRS-1 and IRS-2 (enhanced serine/threonine and reduced tyrosine phosphorylation) in Nrf2 knockout mice reduce their association with the receptor [64]. In vivo, tyrosine phosphorylation of the IRS-1 and IRS-2 in response to activation of the insulin receptor was strongly reduced in the regenerating liver of Nrf2-deficient mice [65]. Consistent with these data, association of PI3K with IRS-1 in response to insulin was reduced in Nrf2-deficient hepatocytes in vitro and reduced association of these proteins was also seen in hepatectomized liver of these mice in vivo. One of the inhibitory IRS serine/threonine kinases may be JNK, which is known to be activated by ROS [66] and which showed enhanced and prolonged activation in the injured liver of Nrf2-deficient mice [63]. Moreover, activation of the PI3K/Akt pathway was strongly impaired in the absence of Nrf2 [63]. Nrf2 knockout mice showed strongly reduced activation of Akt and its targets, GSK-3β, p70/S6 kinase and Bad were also reduced [67]. Thus, the strong insulin-mediated activation of Akt was no longer observed in cells from Nrf2-deficient mice [65].

Obesity is believed to be an important risk factor for the pathogenesis of T2DM. Nrf2 inhibits lipid accumulation and oxidative stress in mouse liver after feeding a high fat diet, probably by interfering with lipogenic and cholesterologenic pathways [68]. Moreover, the inhibitory effect of bardoxolone methyl, a synthetic oleanolic triterpenoid and an inducer of Nrf2, on lipogenic gene expression was significantly reduced in Nrf2-disrupted mice [69].

Further studies are needed to define the role of Nrf2 in diabetes and clarify the mechanisms that are responsible for the development of this disease and its complications and even for the insulin resistance and reduced GSIS.

7. Candidates of natural or synthetic compounds that could directly activate Nrf2 and decrease oxidative stress related to T2DM

Multiple natural or synthetic antioxidants have found to be potent Nrf2 activators, which could decrease oxidative stress related to T2DM, such as oleanolic acid (OA) and bardoxolone methyl.

OA is a natural triterpenoid, which has been used in Chinese medicine for the treatment of liver disorders for many years. OA exists largely in food products (vegetable oils), and is a constituent of the leaves and roots of Olea europaea, Viscum album L., aralia chinensis l. and many others. Our previous study has shown that OA is a potent antioxidant which could activate Nrf2 and protect hepatocytes against tert-Butyl hydroperoxide (tBHP)-induced oxidative stress [39]. The phosphorylation of Akt and extracellular signal-regulated kinase (ERK) were involved in the antioxidant activity of OA [39]. Our previous results have also showed that the administration of OA could significantly reduced STZ-induced increase of blood glucose in rats [70] It has been also shown that OA could inhibit tBHP-induced insulin resistance in hepatocytes [70], in which process the phosphorylation of ERK and the protective effect on mitochondrial function may be involved. We hypothesize that, through the activation of Akt and ERK, OA activates Nrf2 and plays a potent antioxidant activity, and thus may become a potential pharmacological agent for the treatment of DM.

As mentioned above, bardoxolone methyl is a synthetic oleanolic triterpenoid that is another potent known inducer of the Keap1/Nrf2 pathway and works to suppress both oxidative stress and inflammation [71]. Bardoxolone methyl has entered clinical development for the treatment of moderate to severe chronic kidney disease in T2DM patients. Bardoxolone methyl treatment may reduce stage of chronic kidney disease and improve estimated glomerular filtration rate (eGFR) in kidney function in T2DM patients, which were presented in an oral presentation at the American Society of Nephrology (ASN) Renal Week Conference in Denver [72]. Bardoxolone methyl may be a promising agent for the treatment of chronic kidney disease and other complications in T2DM.

8. Cross-talks between Nrf2 and several diabetes-related factors

8.1. Nrf2 and PPARγ

The family of peroxisome proliferator-activated receptors (PPARs) is thought to be involved in the control of fat cell development and glucose homeostasis [73]. PPARγ, which is the target of a new class of insulin-sensitizer agents (thiazolidinediones, TZDs), is expressed predominantly in adipose tissue, resulting in differentiation and triglyceride synthesis [74]. These agents work primarily by binding to nuclear PPARγ [75], thus improving insulin sensitivity, reducing triglyceride levels and decreasing the risk of atherosclerosis in diabetic patients and also exerting direct effects on vascular wall cells [76]. In deed, a number of evident have showed that PPARγ ligands have antioxidant activity and promote the expression of antioxidant enzymes [77]. It has been reported that, in response to the PPARγ agonists, Nrf2 was activated to bind to ARE and stimulate the expression of the GSTA2 gene [78]. TZDs have also been reported to mediate their antioxidant effects through inhibiting nitric oxide synthase, thereby decreasing peroxynitrite and superoxide production [79].

8.2. Nrf2 and PGC-1α

Peroxisome proliferator activated receptor (PPAR) γ coactivator 1α (PGC-1α) is a large protein with a number of functional domains that could bind various protein complexes, the N-terminal of which contains an activation domain, which interacts with CBP/p300 [80]. PGC-1α can activate most nuclear receptors and also function as a coactivator to many transcription factors [81]. PGC-1α is a critical regulator of metabolism that links metabolic activity to relevant environmental stimuli in multiple pathways, including those responsible for adipogenesis, gluconeogenesis, myogenesis and mitogenesis [82]. Furthermore, PGC-1α is emerging as a key transcriptional regulator of antioxidant defense system and coordinates the expression of many antioxidant programs in response to oxidative stress [41, 83]. In deed, PGC-1α has been reported to control many aspects of oxidative metabolism, including mitochondrial biogenesis and respiration through the coactivation of many nuclear receptors and factors outside the nuclear receptor family. ERRα, Nrf1 and Nrf2 are key targets of the PGC1s in mitochondrial biogenesis [84]. PGC1α and β are induced when cells are exposed to oxidative stressor, such as H_2O_2. In fact, experiments with RNAi for the PGC1α show that the ability of ROS to induce the antioxidant program depends on the PGC1α [84] , indicating that PGC-1α is a key regulator of these protective responses. In the PGC-1α-/- mouse, basal levels of CAT, as well as CuZnSOD and MnSOD (but not Gpx1) appear decreased [85]. Taken together, PGC-1α and Nrf2 are complementary and overlapping regulators of the antioxidant defense system and cooperatively regulate several enzymes in the enzymatic antioxidant defense system. So far, the majority of antioxidant enzymes found to be regulated by PGC-1α locates or is activated in the mitochondria. However, a direct molecular interaction between PGC-1α and Nrf2 has

not yet been characterized. PGC-1α promoter contains an ARE [41] , although it is not known whether it is functional. Even without a direct interaction between Nrf2 and PGC-1α, it also seems probable that the activation of one gene may regulate the expression of the other via redox signaling. Both of the two genes can be induced by ROS and increase downstream anti-oxidant enzymes, which in turn reduce ROS. Therefore, the diabetes therapeutic strategy that utilizes Nrf2 or PGC-1α must be able to reduce ROS, only to a suitable level that restore redox homeostasis to the cell but do not affect the normal signal in which certain degree of ROS is required [86].

8.3. Nrf2 and PI3K/Akt

Oxidative stress has been implicated in the impairment of PI3K and Akt signaling during T2DM. Also, PI3K/Akt pathways are reported to be involved in HO-1 expression and in Nrf2-dependent transcription [87]. And the PI3K/Akt/Nrf2 signaling has been found to be responsible for insulin-induced HO-1 and GCLC expression [57,58]. The pathway of PI3K is activated by oxidative stress, leading to rearrangement of actin microfilaments and then depolymerization of actin which causes a complex of Nrf2 bound with actin to translocate into the nucleus [88]. Ginsenoside Rb1 was found to activate PI3K/Akt pathways and the use of specific inhibitors for PI3K/Akt pathways confirmed the involvement of PI3K/Akt in Ginsenoside Rb1-induced HO-1 expression, Nrf2 nuclear translocation, transcriptional activity and cytoprotection [87]. Moreover, Eckol attenuates oxidative stress by activating Nrf2-mediated HO-1 induction via ERK and PI3K/Akt signaling [89].

8.4. Nrf2 and GSK-3β

In recent years, it has been shown that there is a loss in oxidative stress tolerance with aging which is linked to a parallel increase in glycogen synthase kinase-3β (GSK-3β) activity. GSK-3β is a serine/threonine kinase involved in energy metabolism, neuronal cell development and midline development [90]. In addition to regulating PGC-1α protein stability, GSK3β also seems to negatively regulate Nrf2 in response to oxidative stress, although the precise mechanisms are still to be cleared [41]. H2O2 induces tyrosine 216 phosphorylation of GSK-3β resulting in its activation. GSK-3β maintains Nrf2 within the cytosol in mouse N2A neuroblastoma cells [91], excludes Nrf2 from the nucleus and inhibits the transcriptional activity of Nrf2 [91]. Lithium, a GSK-3β inhibitor, promotes Nrf2 transcriptional activity towards an ARE luciferase reporter and cooperated with sulforaphane (SFN) to induce this reporter and to increase the protein expression of HO-1. Conversely, ARE activation by SFN is attenuated by over-expression of active GSK-3β. Prolonged oxidant exposure activates GSK-3β and limits the antioxidant cell response by keeping Nrf2 off the nucleus. Besides, GSK-3β reversed the cytoprotective effect of Nrf2 in the presence of H_2O_2. Previous data reveal that GSK-3β is upstream to Fyn, which phosphorylates Tyr-568 of Nrf2 that regulated nuclear export of Nrf2

[92].

These findings support that there are axises between Nrf2 and PPARγ, PGC-1α, PI3K/Akt and GSK-3β, which could be used as a pharmacological target in prevention of oxidative stress related diseases, especially diabetes. The cross-talks between these important factors implicate that Nrf2 is a pivotal determinative factor which determines the cell fate in response to oxidative stress or certain level of ROS (Fig. 5).

9. Conclusion

The worldwide prevalence of T2DM makes it the most serious healthcare concern now and in future. Knowledge gained in understanding the complex cellular and systemic processes of T2DM provide essential insight into the pathogenesis of diabetes and its complications. Although the clear mechanism of T2DM is still to be elucidated, it has been well established that ROS derived from multiple sources plays a causal role in multiple types of insulin resistance [10] and contributes to β cell dysfunction thus enhances the development and progression of T2DM and its related contributions [5]. It is incomprehensible that the detrimental ROS also plays a substantial role in the normal insulin signaling transduction and GSIS in β cell. Moreover, the use of antioxidant become more complicated [93], because of the dosage of antioxidant must be appropriate to ensure that ROS level is decreased but not to the level that is not enough for normal physiological function. But it is too difficult because of the non-specific characteristic of most antioxidants, which has a potential to be “too much of a good thing”, producing unexpected effects. These discoveries force us to re-recognize the role of ROS under physiological and pathological conditions in a more broad way.

Therefore, the following hypotheses may help us understand the biological effect of ROS: (a) Concentration-dependent biological effect of ROS. Relatively low (physiological) level of ROS is a required signal molecule, which is essential in the insulin action and β cell function and many other physiological functions, but under oxidative stress, when the body’s antioxidant defense is not able to counteract excessive ROS, ROS become a detrimental agent, which will disturb the insulin signal and GSIS, leading to the occurrence of oxidative stress-related disease, including T2DM. Under physiological conditions, organisms have the ability to control the ROS under a physiological level. When this ability is missed, disease will come to exist; (b) The paradoxical regulation of Nrf2. Nrf2 is the central transcription factor for regulating redox balance. ROS not only can activate Nrf2 through regulating Keap1 and Nrf2 itself, but also can inactivate Nrf2 through the activation of GSK-3β or other potential kinases. And this may contribute to the paradoxical biological effect of ROS. The ability of Nrf2 to respond to the changes of ROS concentration and to correlate with a series of diabetes-related regulators determines Nrf2 as a pivotal factor in the development of T2DM and a novel potent target for the treatment of T2DM; (c) ROS acts as both a signal and a stressor in a broad way.

The role of ROS in signal transduction is not very specific. ROS could oxidize a number of signals and/or kinases and thus affect many pathways that could not clear easily; (d) Interactions of different kinds of ROS. For example, H_2O_2 could react with NO, which is benefit for the health, generating •ONOO− which is detrimental to cells. Therefore, the interactions of different ROS may contribute to the bilateral effect of ROS; (e) Compartment of cells. Cells have compartment to limit different ROS in a local region. ROS only could play as a signal locally because of its high reactivity. But when ROS is excessive, it can extend without being cleared, leading to oxidative stress.

Taken together, these hypotheses may have a common center-Nrf2, which is in the point of decision about what role ROS will play. ROS activate multiple signaling pathways in cells that determine whether they will ultimately tolerate or succumb to this aggression. Nrf2 appears to be one such pathway that will determine the destiny of ROS [94,95] due to the tightly regulation of redox homeostasis by this transcription factor. The fact that oxidative stress activates contradictory signaling pathways either to survive or die implies that there must be a complex cross-talk between these opposite signals that determine the destiny of cells. Consistent with these hypotheses, PPARγ, PGC1α, PI3K/Akt and GSK-3β are critical regulators of cell fate, survival or death, in normal and pathological physiology [96]. Accumulating evidence suggest that there are important cross-talks between Nrf2 and these factors, on regulating antioxidant enzymes and the development of diabetes. Therefore, these evidence suggest that Nrf2 may be a critical element in taking survival and death decisions when cells are exposed to an oxidant environment [91]. We propose that Nrf2-mediated antioxidant response plays a paradoxical role in insulin action and insulin secretion (Fig.5). On the one hand, it protects insulin signal and β-cells from oxidative damage and possible cell death, thus minimizing oxidative damage-related impairment of insulin action and insulin secretion. On the other hand, situations leading to chronic induction of endogenous antioxidants mediated by Nrf2 due to oxidative stress may blunt endogenous ROS signaling, resulting in reduced insulin signal and GSIS. In conclusion, enhancing GSIS and insulin sensitivity through the regulation of Nrf2 levels is a potential avenue for developing new therapeutics (Fig. 5). Nrf2 may become a promising target for the treatment of T2DM, compared with nonspecific antioxidants. The effectiveness of bardoxolone methyl is a potent example. By inhibiting Keap1, it induces the activity of Nrf2 to suppress ROS formation and ROS-driven inflammation. The future agents could take similar strategy to directly to Nrf2, which could help provide a perspective for the Nrf2 as a potential target. However, further studies are needed to provide more specific answers in this important area of research.

10. Figures

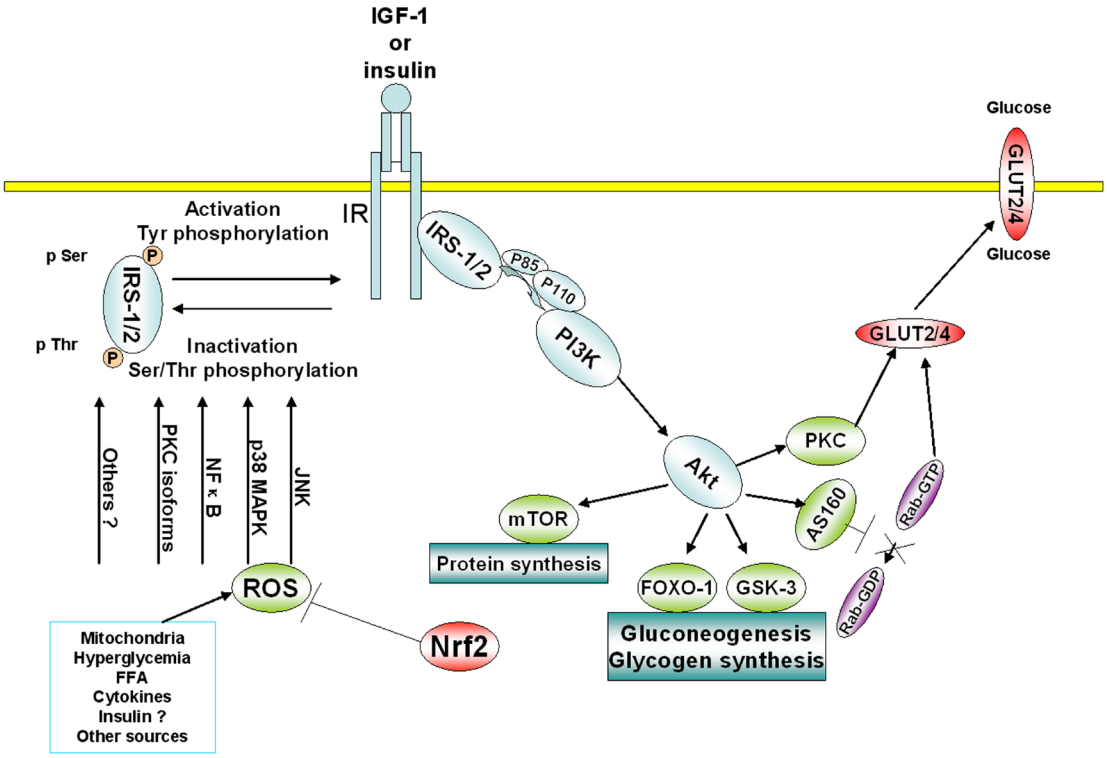

Figure 1: Schematic representation of insulin signal and the effect of ROS.

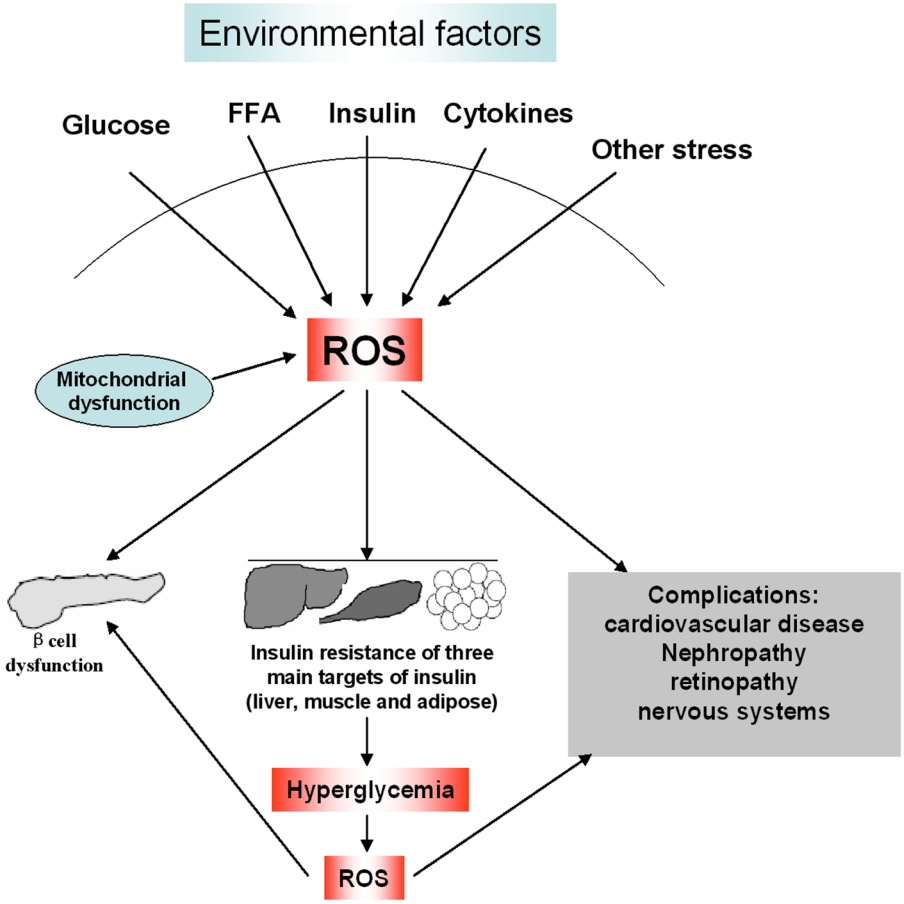

Figure 2: The role of oxidative stress in the development of T2DM.

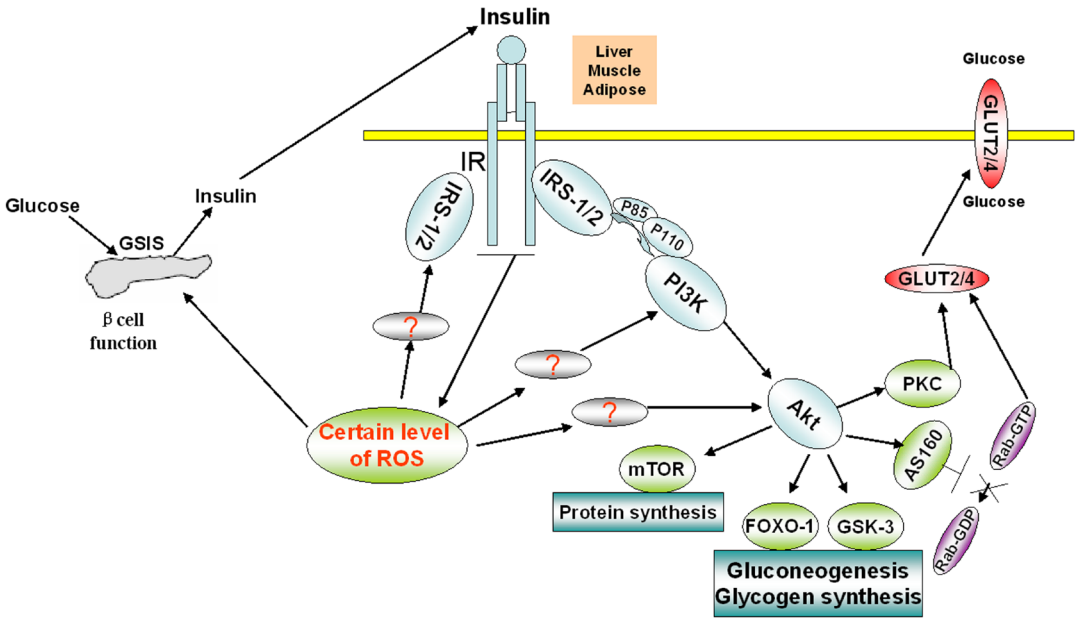

Figure 3: The role of ROS signaling in the insulin signal and GSIS in β cell.

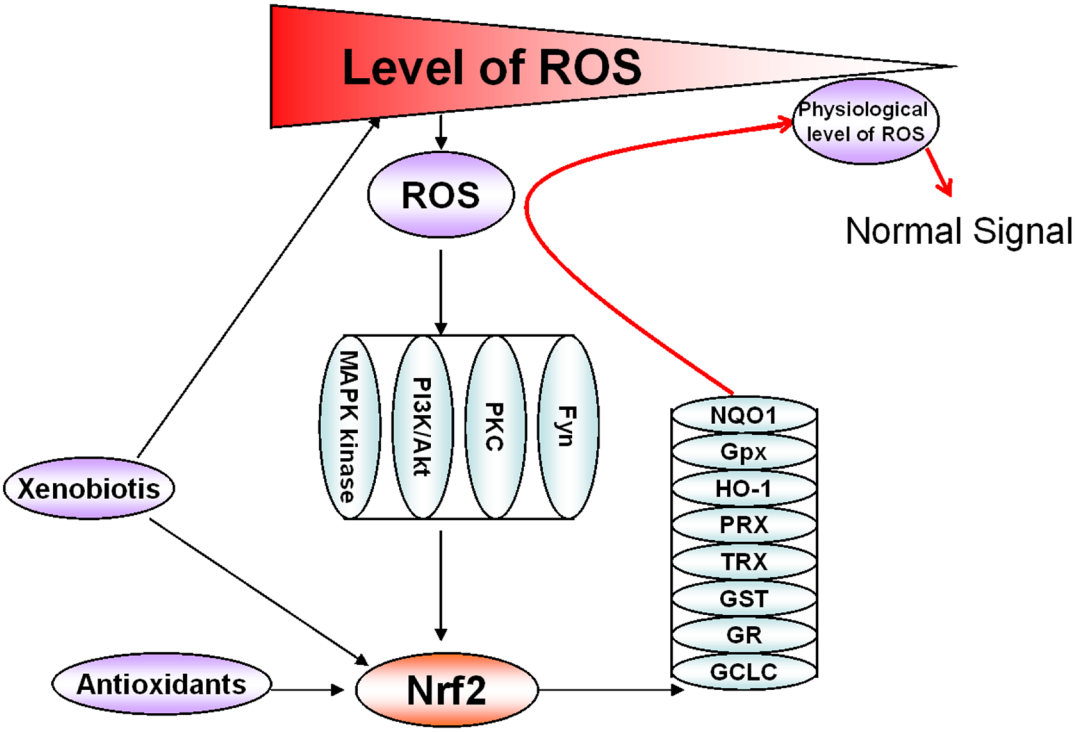

Figure 4: Regulation of redox balance by Nrf2.

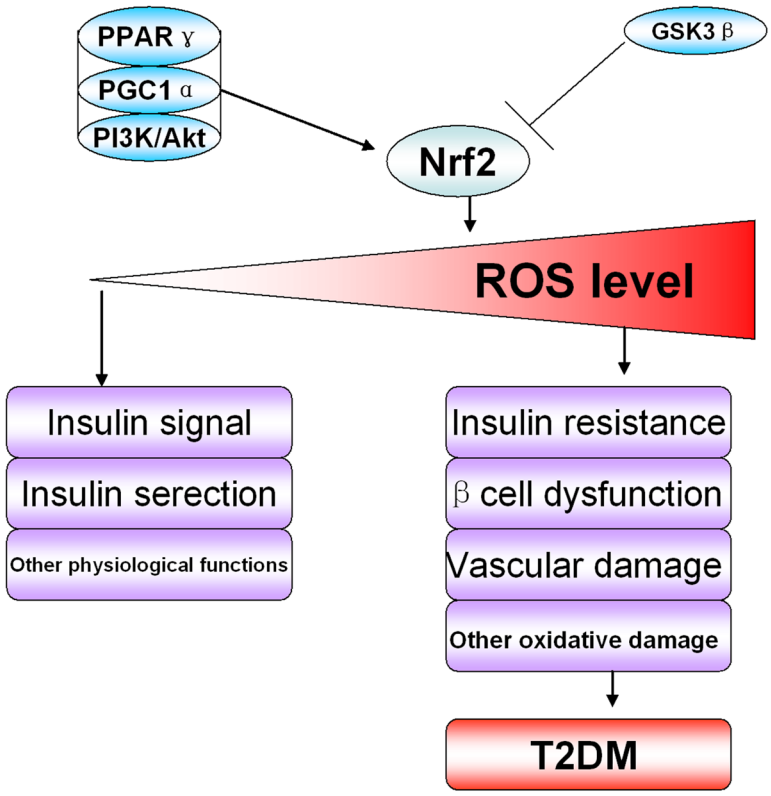

Figure 5: Regulation of insulin sensitivity and β cell function by Nrf2 through manipulation of ROS level.

11. Acknowledgments

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Chapter 2

Oral Agents for Type 2 Diabetes Mellitus

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1. Introduction

Recent advances in the pathophysiology of Type 2 Diabetes have made tremendous progress in the development of new treatment modalities for Diabetes. These new treatment regimens have allowed clinicians to tailor fit medications according to their patients' diabetes profile and phenotype.

In my review publication on pharmacologic treatment options for Type 2 Diabetes published at the Mayo Clinic Proceedings in 1996, Tan et al emphasized that only three classes of oral medications were then available for our patients namely sulfonylureas, biguanides and alpha glucosidase inhibitors [1]. Twenty years later, significant changes in the understanding of diabetes have resulted in newer and safer medications for our patients. The Ominous Octet physiology of Diabetes [2] have advanced our way of treating diabetics with monotherapy to early combination therapy using different agents acting on different mechanisms.

2. Sulfonylureas

This class of drugs has been around us for the longest. They mainly act by stimulating insulin secretion [3]. Sulfonylureas are considered potent and fast enough to lower blood sugar level and can lower the A1c by 1-2%. This is one drug that allows one to see major changes in the level of blood sugar in a short period but the major side effect of this potency is hypoglycemia. It is recommended that these agents are to be used with caution for those at risk of hypoglycemia especially among elderly and among patients with liver or renal dysfunction. Due to the risk of hypoglycemia and weight gain, it is no longer considered as the first line of treatment as recommended by the American Association of Clinical Endocrinologist (AACE)

Treatment Guidelines for 2016 [4].

The major route of elimination is renal and therefore it is contraindicated in patients with renal insufficiency. Certain sulfonylureas like glipizide and gliclazide (available only in Europe) however are excreted as inactive metabolites in the kidney and therefore are preferred in patients with mild renal insufficiency. The major advantage of sulfonylureas is the cost and is readily available in generic forms and therefore used extensively as first line agents for the treatment of diabetes in developing countries. If one prefers this drug for their patients, it is always advised that patient education and awareness of their side effects should be emphasized to guide patients on what to do if hypoglycemia occurs.

3. Biguanides

The only approved biguanide in the market is Metformin. It is known that its principal target organ is the liver and acts by regulating hepatic glucose output in both the fasting and postprandial state. It is the current first line of therapy in almost all guidelines due to its long safety record of no hypoglycemia and no weight gain [5]. Major pleiotrophic effects of this drug include remarkable cardiovascular safety [6], without increasing islet insulin secretion, and possible benefits in reducing cancer risk and improving cancer prognosis [7-8]. It is recommended as initial monotherapy or in combination with other agents and has been shown to lower A1c by 1-2%. Contraindication to the use of metformin is renal dysfunction with an estimated Glomerular Filtration Rate (eGFR) of below 30 ml/min/1.73m^2. Starting treatment with the drug in patients with an eGFR between 30 and 45 mL/min/1.73 m^2 is not recommended [9]. This drug can be used once or twice daily and comes either in the Intermediate release or Extended release form.

4. Alpha glucosidase inhibitors

Considered one of the weakest agents in the treatment of diabetes that can lower A1c by only 0.5% [10]. It's effect is mainly to lower the post prandial blood sugar. The agents act by inhibiting the cleavage of disaccharides to monosacharides on the brush border membrane of the intestinal epithelial cell. The principal side effect is increased flatulence from the undigested carbohydrate. It is not widely used worldwide.

5. Thiazolinediones

The only drug with an effect in improving peripheral insulin sensitivity and in reducing insulin resistance allowing the endogenous insulin to work more effectively. It has also been shown to help protect the cells in the pancreas, allowing them to carry on producing insulin for longer period to time. It works by binding to and modulating the activity of the nuclear transcription factors Peroxisome Proliferator Activated Receptors (PPARs) particularly PPAR

–gamma [11].

The downside in the use of these agents is the side effect of fluid retention, resulting in peripheral edema and risk of heart failure [12]. Other reported side effects of these agents include increase fracture risk [13] and some studies suggesting a small but dose and duration dependent increase in the rate of bladder cancer especially among patients taking pioglitazone but an overall reduction in the rate of other cancers like breast and colon.

6. Incretins

Incretins are peptides that are released from the gut in response to a meal [14]. They stimulate insulin production by the beta cells mainly via the substance called Glucagon-like peptide 1(GLP-1). Likewise GLP1 has been shown endogenously to stimulate glucagon production and also delays gastric emptying. The stimulation of insulin by this product has a safety net in the sense that it is abolished once glucose level approaches the hypoglycemic level. Endogenous GLP1s however are rapidly degraded by an enzyme called dipeptidyl peptidase 4 (DPP4). Oral incretin therapies are therefore developed as DPP4 inhibitors allowing levels of GLP1 in circulation longer to exert its glucose lowering effect [15].

There are now several DPP4 inhibitors in the market. Currently Linagliptin, Saxagliptin, Sitagliptin, Alogliptin and Vildagliptin are approved for use in different parts of the world. They can lower the A1c by as much as 0.7%. Due to its low risk of hypoglycemia and weight neutrality, the use of these agents has increased over the years. Except for linagliptin, DPP4 inhibitors dosing should be adjusted once the eGFR reaches 30-60 ml/min/1.73m2. Caution likewise should also be exercised for patients with history of pancreatitis [16].

7. SGLT2 inhibitors

It is now known that that kidneys play a very important role in the pathophysiology of Diabetes. Increasing levels of blood sugar result in increasing levels of glucose in the urine due to the renal threshold for glucose absorption in the kidney tubules which is 180 grams per day is being exceeded [17]. SGLT2 is a high capacity low affinity transporter of glucose and sodium located in the proximal tubule and is responsible for the absorption of 90% of glucose [18]. By inhibiting the action of these SGLT2s therefore result in the inhibition of glucose reabsorption resulting in an increase in glucose excretion by as much as 70 grams per day. This action offloads the pancreas of work and can help lower blood glucose and lose extra calories that can result in weight loss by as much as 2-3 kgs [19]. The most common adverse events include urinary tract Infection and mycotic genital infections related to glucosuria [20].

For now, there are three approved SGLT2 inhibitors in the market namely: Canagliflozin, Dapagliflozin and Empagliflozin. They can be used as monotherapy or in combination with

other agents. Caution however for very rare occurences of Euglycemic Diabetic Ketoacidosis reported in patients with diabetes who may have the following triggering factors: intercurrent illnesses, reduced food and fluid intake, reduced insulin doses and a history of alcohol intake [21].

8. Clinical practice setting

The American Diabetes Association and the American Association of Clinical Endocrinologists have updated guidelines in the use of oral agents in the treatment of Diabetes [22]. Both guidelines strongly emphasized on individualized approach based on patient characteristics, age, risk of hypoglycemia, duration of disease and presence of co-morbidities. Likewise approach to the use of different agents should be made to avoid hypoglycemia and weight gain.

Depending on the duration and onset of the disease, patients are started on monotherapy or initial combination therapy to achieve the desired A1c goals. Patients however should be included in the decision making and plan of therapy. Compliance to medications and follow-up requires physicians to practice Empathy [23]. Taking time to communicate with the patients including understanding how diabetes progress with time will give us the assurance of better long term outcome and better chances of preventing diabetes complications and disability.

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Chapter 3

The old and the new risk factors for chronic renal disease in type 2 diabetics

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Abstract

The pathophysiological mechanisms associated with chronic renal disease in type 2 diabetic are complex and still remain to be fully understood. It is well known that there are traditional factors responsible for kidney disease such as poor metabolic control, time of evolution of diabetes, age, gender, uncontrolled hypertension, ethnical background and genetic factors. Nevertheless, recent evidence also points to non-traditional risk factors that might trigger glomerular barrier changes, leading to an increase durinary album in excretion along side with the progression of diabetic nephropathy to end-stage chronic kidney disease. Among these factors are inflammation and oxidative stress; alterations in mineral metabolism and homeostasis might also contribute to the clinical manifestation and/or progression of diabetic nephropathy.

Our proposal is firstly to discuss the traditional risk factors associated pathogenesis and progression of chronic kidney disease associated with diabetes and secondly we will consider the new risk factors or new signs including the Klotho-FGF23 axis, magnesium and vitamin D.

Keywords: Chronic Kidney Disease; Diabetes; Mineral Metabolism

1. Introduction

Diabetes mellitus is one of the major epidemics in the modern world, with a world wide increase in incidence by around 50% over the past 10 years [1].

Diabetes is a chronic and multifactorial disease. There are 2 major forms of diabetes that vary in their aetiology, type I and type II, although both are clinically characterised as hyperglycaemia due to chronic and/or relative insulin in sufficiency [1].

Type II diabetes mellitus is characterised by insulin resistance concomitant with insulin deficiency. The increasing peripheral resistance to insulin is off set over the course of years by an increase of insulin secretion by the pancreatic β-cells, the biological source of this hormone. The progressive decline of pancreatic function as age advances and chronic hyper stimulation result in an increasing hyperglycaemic state [2].

The most devastating consequences of diabetes are vascular complications, which can be organised into micro vascular and macro vascular and can occur in both forms of diabetes (type I or type II). The most common microvascular complications are nephropathy, retinopathy and neuropathy, with myocardial in farction and stroke being the most frequent macrovascular complications [1].

The aim of this work is to summarise current scientific knowledge on factors that contribute to the urinary excretion of albumin in type II diabetics.

2. Old Risk Factors for Chronic Renal Disease in Type 2 Diabetic Patients

Diabetic nephropathy (DN), besides being a major risk factor for cardiovascular complications perse, is the major cause of end-stage renal disease in Western societies.

DN develops in 35-40% of diabetic patients as the result of intra renal metabolic, hemodynamic and structural changes [1,2]. DN is a complex phenotype caused by the combined effects of susceptibility alleles and environmental factors which contribute to poor glycaemic-control and hypertension [1].

The nephropathy does not necessarily develop in a significant proportion of diabetic patients, suggesting the involvement of specific genes [1].

2.1. Pathophysiology of Diabetic Nephropathy

It is established that hyperglycaemia is the triggering factor of tissue damage in diabetes [1,3].

But why are only some cells in the body affected? The answer is obtained by studying the mechanisms of glucose transport in the cells of the different tissues. In contrast to tissues damaged by hyperglycaemia (capillary endothelial cells in the retina, mesangial cells in the glomerulus, neurons and Schwann cells in the peripheral nerves), the other cells have the ability to regulate the transport of glucose to inside the cell, reducing it when exposed to hyperg-

lycaemia and consequently keeping intracellular glucose levels constant [1,3].

The glomerulus is the location of histological lesions associated with DM, where mesangial cell proliferation, the excessive production of extracellular matrix (fibronectin, laminin, collagen type IV), occurs due to increased levels of intracellular glucose.

The mechanisms associated with DN are not fully understood, and many of the molecular factors associated with the renal lesion are under investigation.

However, it is known that there are four mechanisms responsible for the onset of DM complications that are triggered by the increase of intracellular glucose.

These mechanisms are:

Increased flow through the poliospathway, with consequent increase of sorbitol in the tissues, including the renal tubules and glomeruli. This increase in sorbitol causes tissue lesion by changing the cellular osmo regulation. This is the primary mediator of protein kinase C (PKC) cellular activation [3].

The increase in the non-enzymatic advanced glycosylation end products (AGEs) causes an increase in plasma proteins and extracellular matrix. When AGEs bind to specific receptors, identified in the macrophages of endothelial and mesangial cells, they induce the synthesis and secretion of cytokines (ILTNF-α, IL -1,IL-6) and insulin growth factor (IGF1), stimulating mesangial cells proliferation and collagen IV production. Moreover, by cross linking with collagen, AGEs can induce an increase in extracellular matrix synthesis by stimulating growth-factors (TGF-β1,CTGF,VEGF,PDGF) [3-5];

Increase of the flow through the hexos amine pathway, causing changes in gene expression of glomerular cells and endothelial cells [3].

These mechanisms are, among others, responsible for the changes in the glomerular filtration barrier whose manifestation is the emergence of proteinuria.

High intracellular glucose concentrations in susceptible tissues lead to the dysfunction of several cell signalling pathways with the consequent increase in the mitochondrial production of ROS (reactive oxygen species) that damage the cell itself. This fact explains the microvascular damage caused by hyperglycaemia [1,3].

Regarding the macrovascular damage hyperglycaemia is not a major direct determinant, being insulin resistance the “culprit” of this phenomenon. Insulin resistance promotes an increase in the release into the blood stream off ree fattyacids by the adipocytes, which will be oxidized by the mitochondria of ROS-producing endothelial arterial cells. Again it is the increase in ROS production that causes cellular damage [1,3] (**Figure1**).

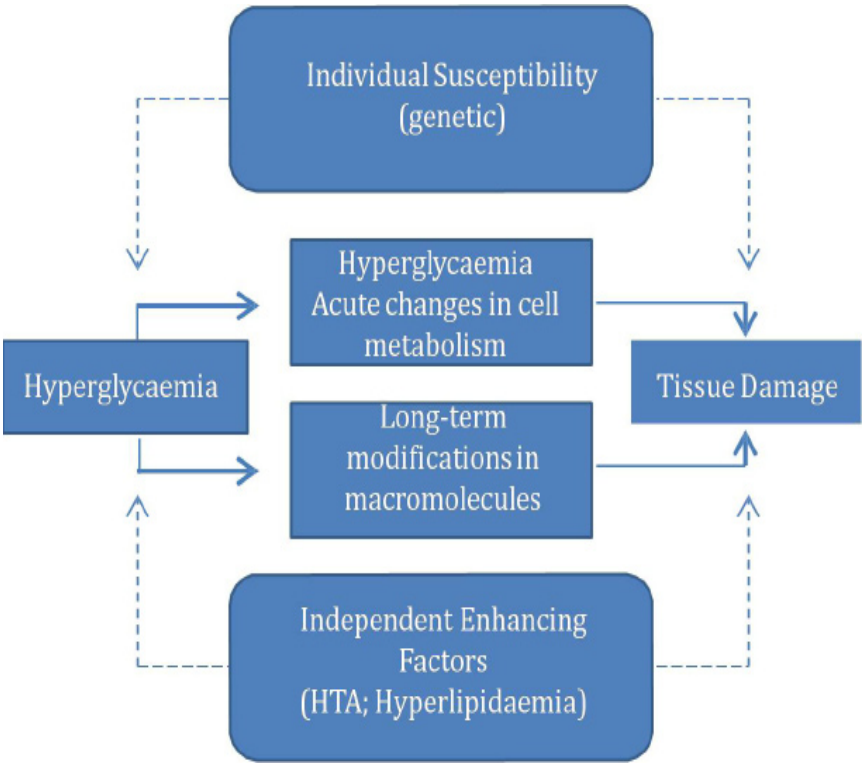

Figure 1: Mechanism of tissue damage resulting from hyperglycaemia.

Several authors have found that the excessive production of advanced glycation end products and free radicals resulting from chronic hyperglycaemia with consequent imbalance of the anti-oxidative mechanisms culminates in endothelial dysfunction [4-5]. Oxidative stress has been increasingly recognised as an important agent causing kidney damage. Infact, it is thought that the underlying factor of renal damage is a disturbance of the balance between oxidative and anti-oxidative mechanisms and that this alteration precedes renal injury [4,6]. It is also believed that the oxidative mechanisms gradually increase and in parallel with the progression of the renal disease [6-7].

In addition to direct tissue damage, these glycation metabolites promote the increase of angiotensin II, apro-fibrotic, pro-angiogenic and pro-inflammatory agent that plays an important role in the renal diabetic pathology [8-10]. Angiotens in II it is the main mediator of TGFβ1 and connective tissue growth factor (CTGF) production at the level of mesangial and tubular cells, leading to an increased production of extracellular matrix and contributing to the development and progression of glomerulosclerosis and tubulo interstitial fibrosis and sclerosis [10,11]. Moreover, it was described the possible directaction of saturated fatty acids on podocytes contributing to their dysfunction and resulting in albuminuria [12] (**Figure 2**). These damaging stimuli manifest themselves, at a nearly stage, by an increase in systemic blood pressure and consequent increase of glomerular pressure and glomerular hyperfiltration [13]. The kidney under goes hypertrophy and hyperplasia. Glomerular and tubular hypertrophy, thickening of the glomerular basement membrane and mesangial expansion are identified [1]. This transformation leads to an increase of the glomerular filtration rate (GFR) and increased filtration of glucose, fattyacids, proteins, and aminoacids, growth factors and cytok-

ines, responsible for the dysfunction of anti-oxidative mechanisms, inflammation and fibrosis [1].

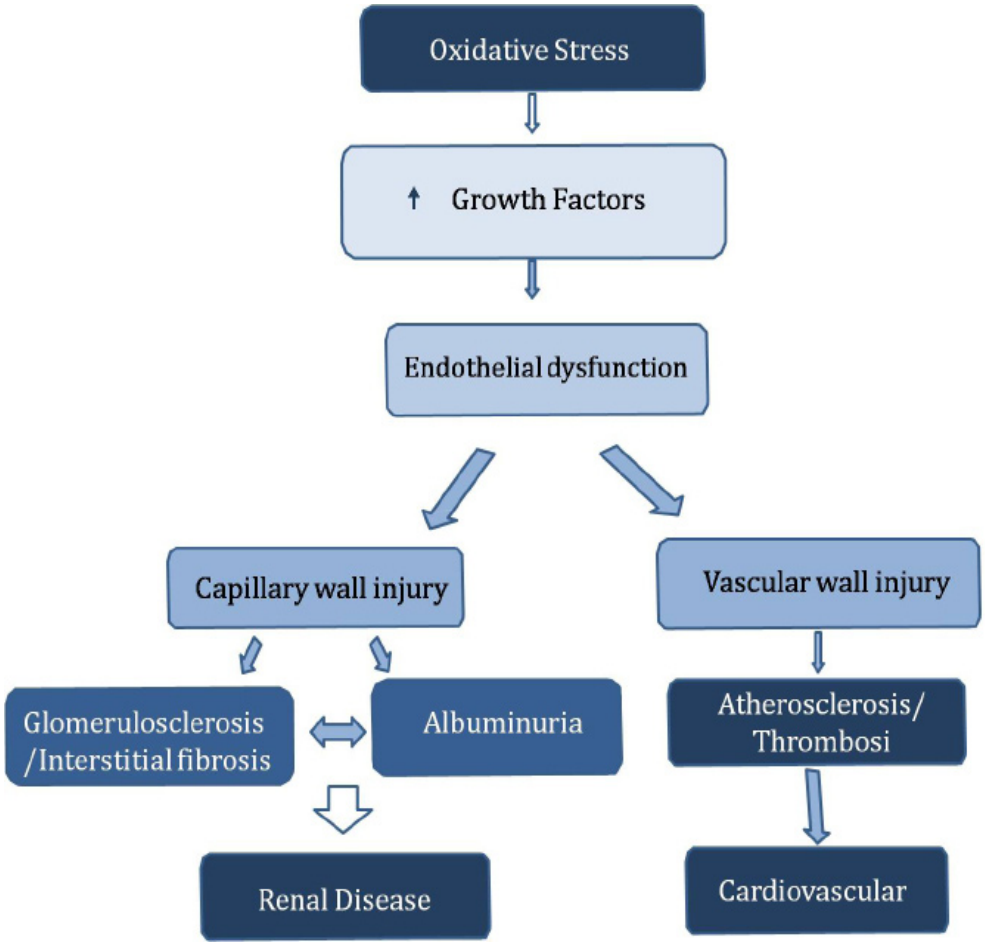

Figure 2: Pathophysiology of oxidative stress in renal and cardio vascular disease

The renal repercussions induced by these damaging stimuli manifest themselves, at a nearly stage, by an increase in systemic blood pressure and consequent increase of glomerular pressure and glomerular hyperfiltration [13]. The kidney undergoes hypertrophy and hyperplasia. Glomerular and tubular hypertrophy, thickening of the glomerular basement membrane and mesangial expansion are identified [1]. This transformation leads to an increase of the glomerular filtration rate (GFR) and increased filtration of glucose, fatty acids, proteins, and aminoacids, growth factors and cytokines, responsible for the dysfunction of anti-oxidative mechanisms, inflammation and fibrosis [1].

The progression to proteinuria and subsequent progressive decrease of GFR reflectsthe chronic damage of the glomerular filtration barrier, in particular the glomerular and podocyte epithelial cells, thickening of the basement membrane and mesangial expansion [1] Extra cellular matrix deposition (tubulo interstitial fibrosis) is hypothesized to be the major determining fact or in the progression of renal disease in diabetes [1].

An underlying inflammatory process is common to all pathophysiological mechanisms. Countless studies back there levant role of inflammation in renal disease and the development of micro albumin uria in the general population [14].

Stuveling et al. demonstrated the association between high CRP (C-reactive protein) values and the change in the relationship between blood pressure and micro albuminuria, i.e. inflammation increases the likelihood of glomerular losses of albuminina situation of increased blood pressure [14]. The role of inflammation in nephropathy had already been pointed out by Navarro et al. who demonstrated a dose-dependent increase of micro and macro albuminuria in type II diabetics in response to raised CRP and TNF-α (tumour necrosis factor alpha) [14-15].

2.2. Risk factors for the development of Diabetic Nephropathy in Type II Diabetics

Genetic susceptibility should be regarded as a non-modifiable risk factor that influences both the incidence and the severity of diabetic nephropathy [16].

The prevalence of diabetic nephropathy also varies with ethnicity. It is relatively high in African Americans, Latin Americans from Mexico, Australian Aborigines and Indo-Asian immigrants in the United Kingdom, when compared with Caucasian individuals [1].

Concerning modifiable risk factors, metabolic syndrome,which of ten precedes the development of type II diabetes, is characterised by several metabolic aterations that contribute to the development and/or progression of diabetic nephropathy [17-18].

Also high blood pressure which can be "transferred" to the glomerular system is associated with the progression of renal disease. Several studies have shown that the control of the blood pressure decreases albuminuria and the progression of renal disease [8,12,14].

A high body mass index (BMI) and, therefore, obesity have been strongly associate with an increased risk of developing renal disease in diabetic individuals [8,12].

Dyslipidaemia has a potential role in triggering and progression of chronic kidney disease, particularly low HDL values, hyper triglyceridemia and increased insulin resistance [12,19].

Finally several studies have demonstrated the adverse effects of tobacco on diabetic kidney disease such as worsening of albumin uria, increased risk of developing end- stage renal disease, and decreased survival of haemodialysis patients [8].

2.3. Urinary excretion of Albumin

The term proteinuria refers to the presence of proteins in the urine. Its main component is albumin which, due to its small size, more easily passes through the fenestrated glomerular capillaries. A sarule, this proteinuria reflects changes in renal haemodynamics underlying a pathological modification of the characteristics of the glomerular filtration barrier, particularly the podocytes, glomerular endothelial cells [1].

The presence of proteins in the urine can be classified qualitatively and quantitatively. In relation to the former, proteinuria can be defined as selective or non-selective depending on the type of proteins that are lost in the urine. In selective proteinuria, also called albuminuria, only albumin is lost, where as in non-selective proteinuria there is the loss of albumin as well as other larger plasma proteins, and in this case a greater degree of glomerular damage is implied [1].

Urinary protein losses areal so classified according to the amount of losses. Micro albumin uria is defined as the urinary excretion of small amounts of albumin, more specifically 30 to 300mg/day of albumin. Below this range we have normoalbuminuria and above it there is macro albuminuria and proteinuria when it includes the excretion of proteins other than albumin [20].

Proteinuria is one of several renal disease markers that are used, particularly in subjects with diabetes for screening for diabetic nephropathy [20].

The gold standard for album in quantification is to measure the albumin concentration in urine collected over 24 hours [20]. Given the inconvenience of this type of sample collection, the urinary albumin/creatinine ratio in a urine sample, expressed as mg/g, is typically used. The first urination in the morning is preferably used. The latter method has a sensitivity and specificity level of approximately 95% when compared to the gold standard [20].

The variability obtained led Chenetal. to recommend albumin quantification in 2 to 3 urine collections, atleast two of which are separated by 3 to 6 months [20].

The importance of microalbuminuria is that it is frequently identified in subjects with an established diagnosis of diabetes and it is the simple stand most sensitive prognostic factor to assess the risk of developing nephropathy in diabetics, representing the initial stages of progressive diabetic renal disease [20]. It also allows micro-and macro vascular complications and cardiovascular mortality to be predicted in type II diabetics [20].

Micro albuminuria is often associated with poor glycometabolic control and a higher incidence of chronic complications, such as diabetic retinopathy, peripheral vascular disease and diabetic neuropathy. Moreover, several studies have observed that the increase in the amount of protein in the urine is strongly related to the development of renal and cardio vascular disease, regardless of other markers of structural or functional renal damage [20].

2.4. Diabetic Nephropathy and Albuminuria

Micro albuminuria is a nearly biomarker of renal disease and vascular dysfunction. It is associated with the development of end-stage renal disease, cardiovascular morbidity and increased mortality, in both therenal and the general population [20-23].

The relationship is linear since there is already an increase of the cardiovascular risk for values of urinary albumin excretion that are now a days accepted as normal [20,24]. Currently, it is discussed whether the microalbuminuria value presently accepted, below 30 mg/day, may have a cutoff that is too high due to this apparent increase in cardiorenal risk [25].

Several authors have demonstrated that the extent of microalbuminuria affects the long-term prognosis [26]. Urinary albumin appears to be pro-inflammatory and, therefore, being filtered and reabsorbed at the level of the proximal renal tubules induces there lease of pro-fibrotic cytokines, in particular TGF-β, responsible for the process of glomerulosclerosis and tubulo interstitial fibrosis [24]. Shao et al. also report that although microalbuminuria is the best non-traumatic predictor currently available for early stage diabetic nephropathy, important changes in renal structure may have already occurred before microalbuminuria is present [26].

Karalliedde et al. demonstrated that the reduction of albuminuria led to a delay in the progression of renal disease. In relation to normoalbuminuria and microalbuminuria, there is no evidence that preventive or reversal measures would lead to a better clinical outcome [24].

According to the study by McFarlane et al. the therapies that reduce micro albuminuria reduce the risk of mortality and morbidity, though not primarily directed towards this goal, but the rapies aimed at the reduction of albuminuria seem to have little benefit in this field and have even been associated with an increased number of adverse effects [25].

Classically, diabetic nephropathy is characterised by persistent microalbuminuria that-progresses over time and through the various phases of nephropathy described above. Nevertheless, about 50% of subjects with diabetes and concomitant renal disease do not present the classical changes of diabetic nephropathy in the renal biopsy [25].

In the study developed by Afghahi et al. only one-third of patients who developed renal disease also developed albuminuria [12]. The development of nephropathy with out albuminuria has already been described in subjects with typeII diabetes and without diabetes [27-32]. One of the proposed hypotheses is the fact that the development of renal damage results from glomerular and extra-glomerular changes, where as albuminuria would only result from modifications related to the glomerulus itself [12,28]. More recent studies support this hypothesis, as they have demonstrated a higher prevalence of micro and macro angiopathy in type II diabetics and non-albuminuric nephropathy [12,32-33].

More over, these verity of nephropathy in the biopsy is not always related to the degree of microalbuminuria, especially for values within the micro albuminuria range [25].

Further more, it should be considered that diabetic individuals may suffer from conditions that lead to transitory albuminuria, which are therefore not necessarily related to future-cardio-renal events. Nevertheless, the usefulness of measuring the presence of albumin in urine is extremely high [25].

2.5. Risk factors contributing to urinary excretion of albumin

According to Shao et al. there is a significant correlation between the extent of microalbuminuria in type II diabetic subjects and the markers of oxidative stress studied by this author. Increased synthesis and release of ROS leads to increased oxidative stress and consequent damage of the local tissues, such as proteins [26]. The author highlights the particularly important role of oxidative stress and oxidative damage of lipids in the pathogenesis and progression of microalbuminuria in the early stages of diabetic nephropathy [26].

Chen et al., like many other authors, have observed that ageing, changes in glucose tolerance, insulin resistance, arterial hypertension, obesity and, consequently, high BMI, dyslipidaemia, cardiovascular history and metabolic syndrome are independent risk factors for the presence of albuminuria [12,20,34-36]. Hyperinsulinemia increases glomerular hydro static pressure and renal vascular permeability, aggravating glomerular hyper filtration and enhances renal reabsorption of sodium [35]. Poor glycaemic control, smoking and low HD Lare also risk factors for the development of albuminuria [12].

It is imperative, given the high prevalence of diabetic nephropathy and the absence of detectable symptoms in the early stages of the disease, to study and explore modifiable risk factors, the use of sensitive and specific markers for this condition in order to be able to implement renal disease prevention measures [20]. It is advocated that it is especially important to identify and educate individuals at high risk of developing microalbuminuria not only through diabetes, but also by interacting with other concomitant risk factors [34].

3. New risk factors for chronic renal disease in type 2 diabetics

The central role of albuminuria in the pathogenesis and progression of diabetic nephropathy justifies research for new factors that shape it.

3.1. What is FGF23?

The fibroblast growth factor (FGF) family includes a large number of polypeptides that share a common region. FGF23 is a hormone primarily synthesized by osteocytes and osteoblasts in response to hypercalcaemia and hyperphosphatemia, increased calcitriol and parathyroid hormone. This glycosylated peptide is responsible for phosphate homeostasis. It induces an increase in the urinary excretion of this ion by reducing sodium-phosphate co-transporters in the proximal tubule cells. It also indirectly limits its intestinal absorption [37-40]. FGF23

also plays a keyrole in vitamin D metabolism. It not only inhibits the synthesis of calcitriol in the kidney but also stimulates the catabolism of the active form of vitamin D [37]. Studies conducted on animals have demonstrated a potent inhibition of them RNA expression of 1α-hydroxylase (25(OH)D-1α-hydroxylase) in the proximal renal tubule, responsible for the conversion of vitamin D to its active form, mediated by FGF23 [37-40]. It concomitantly stimulates the expression of 24-hydroxylase (CYP24A1) responsible for the inactivation of calcitriol [37,39-40]. Calcitriol, at the same time, stimulates the synthesis of FGF23 and Klotho expression, increasing Klothom RNA synthesis [37].

The Klotho protein forms a complex with the FGF23 receptor, significantly increasing its affinity for FGF23. This protein is expressed on the surface of various organs, dominating in the kidney, and it seems to be extremely important in the action of FGF23 [37,40]. This "composite" receptor appears to be involved in the pathophysiology of various conditions.

3.2. FGF23-Klotho

The Klotho protein may play a critical role in the survival of subjects with CKD. Its over expression in rats significantly increased their survival [37]. Zanchi et al. demonstrated that ramipril exerts its renoprotective action through a significant increase in Klothom RNA levels and there is no apparent action on FGF23 levels in rats with diabetic nephropathy. Nevertheless, a significantly reduced expression of FGF23 mRNA was documented [40]. This study demonstrated that ramipril prevents the aggravation of renal disease, with significant attenuation of glomerular and tubular alterations, interstitial inflammation and a reduction in proteinuria that reached 55%. It was also showed a positive correlation between renal expression levels of FGF23 and proteinuria, glomerulosclerosis, tubular damage and interstitial inflammation [40].

According to Vervloet et al. there is a significant association between the FGF23 value, proteinuria and smoking in subjects with CKD. Although this relationship is well established, the mechanism by which this relationship develops has not yet been clarified. It is known that tobacco contributes to oxidative stress. It is believed that the FGF23 levels are increased by this means, contributing to the dysfunction of the respective receptors, inducing a state of resistance to FGF23 that is compensated by the increase in its production [41].

Several mechanisms are proposed to justify the relationship between FGF23 and proteinuria. Direct action by FGF23 on the glomerular endothelium is proposed, although there is little scientific evidence supporting this theory. Also proposed is an elevation of FGF23 secondary to a hyperparathyroidism resulting from a vitamin D deficiency. This latter fact would justify proteinuria. Lastly, the most consensual theory is based on the fact that proteinuria *per se* is harmful to the tubular nephron cells, with consequent disruption of the FGF23 operating sites and subsequent compensatory increase in FGF23 secretion. Nevertheless, we cannot

discard the hypothesis that both these disorders, proteinuria and elevated FGF23 levels, may result from the same damaging mechanism to the kidney, such as the aforementioned oxidative stress [41].

FGF23 serum levels are related to the risk of progression of CKD in subjects with diabetic nephropathy in the macroalbuminuria spectrum [38,42]. It is important to clarify whether FGF23 contributes to the progression of nephropathy or if it is just a risk biomarker [38,42].

3.3. FGF23–vitamin D

Calcitriol (1.25-dihydroxyvitamin D_3), a biologically active form of vitamin D_3, has several functions in addition to mineral metabolism. There is evidence that this vitamin is an important immunomodulator, inhibitor of the proliferation of cell differentiation and inhibitor of the renin angiotensin-aldosterone axis [11]. It acts by activating the VDR and modifying the transcription of several genes [11].

Vitamin D synthesis begins in the epidermis, where vitamin D_3(cholecalciferol) is synthesized by the action of UVB rays. It then undergoes two hydroxylations, the first on carbon-25 that occurs in the liver and is not dependent on hormonal regulation and the second hydroxylation is on the first carbon made by 1α-hydoxylase [37]. This enzyme is strongly expressed in the proximal renal tubule. It is the primary source of circulating calcitriol, vitamin D in its biologically active form [37].

Observational studies mention the association between VDR, serum levels of calcitriol, glucose in tolerance and insulin sensitivity [11,43]. It is also accepted that vitamin D, being the main regulator of calcium metabolism, facilitates the production of insulin by acting on pancreatic β cells [11]. This factis strengthened by the identification of VDR in the pancreas and hypovitaminosis D as a riskfactor for the development of type II diabetes and metabolic syndrome as a consequence of pancreatic β-cell dysfunction and increased peripheral insulin resistance [44-45].

Hyperglycaemia decreases the expression of VDR and 1α-hydroxylase in the proximal tubule cells with consequent decrease in vitamin D reabsorption and increased proteinuria [11]. The role of calcitriol in the pathogenesis of proteinuria is mediated through the regulation of cell proliferation, apoptosis, angiogenesis and anti-inflammatory action [11,46]. On the other hand, the combination of hyperglycaemia and decreased VDR results in a greater activation of the renin-angiotens in-aldosterone axis [11].

Recently, the existence of VDR in podocytes was documented. An association between the quantity of this vitamin D and the number of podocytes has also been demonstrated. Considering the important role that the decrease in the number of podocytes has in micro albu-

minuria, this relationship may be potentially important in the prevention and reduction of mesangial cell proliferation and proteinuria [11,47].

There is as low down in the development of glomerulosclerosis and in the progression of albuminuria in rats subjected to subtotal nephrectomy and treated with calcitriol [11,48] and it was also demonstrated that calcitriol reduces mesangial cell proliferation, glomerular hypertrophy, and progression of glomerulosclerosis [11,49].

Similar results were found with paracalcitol: its administration reduced protein excretion in subjects with renal disease and macroproteinuria [50-51] and albuminuria in type II diabetic patients [52]. Several other studies have supported these findings. Therapeutics with calcitriol or VDR activators have shown beneficial effects in reducing the risk of cardiovascular morbidity and mortality, diabetes, auto-immune diseases and cancer in subjects undergoing renal replacement therapy [37]. Paricalcitol in end-stage renal disease promotes a reduction of FGF23 and suppresses the parathyroid hormone [39].

In subjects with CKD, high FGF23 and low vitamin D values are associated with similar adverse reactions, suggesting that the potential toxicity of FGF23 may be partially mediated by there duction in FGF23-induced calcitriol levels [37]. Both disorders, high FGF23 and vitamin D deficiency, are predictors of a rapid progression of renal disease [37] (**Figure 3**).

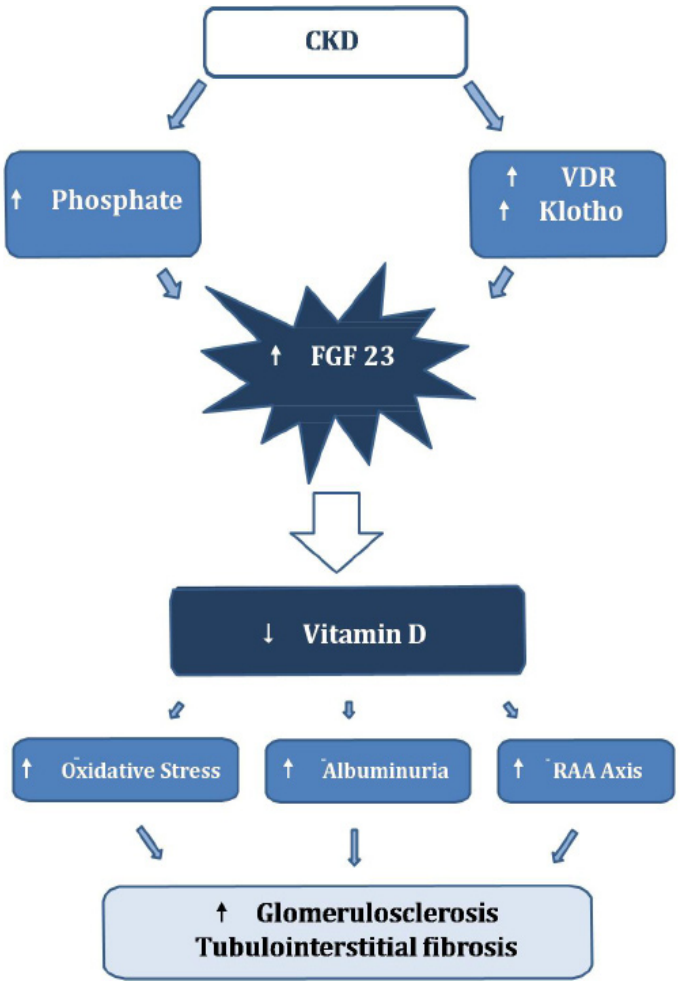

Figure 3: Role of FGF23 in Diabetic Nephropathy

3.4. Klotho and urinary excretion of albumin

Klotho was mainly associated with urinary excretion of albumin. Interstitial inflammation induced by proteinuria may down regulate Klotho expression [53]. There lease of inflammatory cytokines such as tumour necrosis factor (TNF) - like weak inducer of a poptosis'(TWEAK) and TNF-α were responsible for the down regulation of Klotho expression through a nuclear factor kappa-B-dependent mechanism [54]. The observations from More noetal, were supported by the fact that Klotho circulating levels increased in type 2 diabetic patients whose proteinuria was a meliorated with losartan. According to the authors, the use of renin-angiotens in system antagonists in patients with diabetic nephropathy was able to reverse Klotho's down regulation induced by angiotens in II, and therefore retarding disease progression [55].

Another direct mechanism thought to be part of the relationship Klotho/urinary excretion of albumin is the one involving the vascular endothelial growth factor A(VEGF-A). It is believed that VEGF-A plays a pathogenic role indiabetic nephropathy, particularly on angiogenesis and vascular permeability. In their study on diabetic patients, Kacsoetal observed a consistent positive relationship between soluble Klotho and VEGF-A, (both factors having low levels in the presence of microalbuminuria). It is still to be understood if they both respond to the same pathogenic trigger or if it is are active action to the other. Nevertheless, we may hypothesize that the down regulation Klotho/VEGF-A can lead to and urinary excretion of albumin worsening through their impact on endothelial dysfunction [56].

In addition to these mechanisms, Klotho has also an endogenous anti-fibrotic function via antagonism of Wnt/β-catenin signalling, which promotes fibrino genesis. Therefore, it is reasonable to argue that a loss of Klotho may be associated with the progression of diabetic-nephropathy and urinary excretion of albumin worsening by accelerated fibrinogenesis [57-58].

Some studies have also speculated that the correlation between Klotho and urinary excretion of albumin may be due to indirect mechanisms as well. Within the Klotho/FGF-23/ Vitamin D axis, it is already known that low Klotho levels are associated with increased FGF-23 and decreased Vitamin D levels. FGF-23 is then able to indirectly increase proteinuria by diminishing calcitriol synthesis and inducing endothelium dysfunction [59-60]. It is also negatively correlated with Vitamin D levels in diabetic nephropathy patients with microalbuminuria [11,51]. Thus, it is not unreasonable to hypothesize that Klotho and ACR levels might be correlated through indirect, albeit unclear but possibly FGF-23-mediated, mechanisms.

3.5. Magnesium and urinary excretion of albumin

Magnesium is the fourth most abundant cationin ourbody. About 99% is in the intracellular compartment and only 1% in the extracellular fluid [61-62]. It is an essential cofactor in

more than 300 enzymatic reactions, playing an important role in several biological processes, such as cardiac excitability, transmembrane ionic flux, neurotransmitter release, calcium channel regulation and it may even have the role of physiological antagonist of calcium [61,63].

It should also be noted that the total serum value of magnesium does not reflect the intracellular concentration of this ion or its ionized fraction. The small intestine is the primary absorption site of magnesium, while excretion is primarily performed by the kidney, and in healthy subjects serum magnesium levels are extremely constant [62,64].

Some hormones play an active role in the regulation of magnesium metabolism in ourbody, such as insulin [62,64]. Insulin stimulates the transport of magnesium from the extracellular to the intracellular compartment contributing to increase its concentration in the latter, although the specific mechanisms by which this happens are not yet clear [62,64]. The action of insulin on ionic regulation is specific, dose-dependent and independent of glucose up take [62,64]. It is important to note that not only does insulin play an important role in the homeostasis of magnesium, but magnesium itself is an important modulator of insulin action and insulin sensitivity and, therefore, of glucose metabolism [62,64].

Magnesium concentration is determinant in tyrosine kinase phosphorylation of the insulin receptor, as well as other proteinkinases found in the cell membrane and in the endoplasmic reticulum [62,64]. Low concentrations of intracellular magnesium result in impaired tyrosine kinase activity and the consequent development of "post-receptor" insulin resistance associated also with reduced cellular utilization of glucose. In other words, the lower the concentration of magnesium, the less responsive is the cell to stimulation by insulin and a greater quantity of insulin is thus required to metabolize the same amount of glucose [62,64].

These findings confirm the strong dependence on the action of insulin relative to the intracellular concentration of magnesium, and the findings are consistent with the fact that magnesium deficiency is the potential cause and not merely a consequence of peripheral insulin resistance [62].

Following on from what has been stated, an increase in insulin resistance in tissues where the entry of glucose into cells is insulin dependent, such as musculoskeletal, cardiac, and fat, would be expected in the presence of decreased magnesium values. Taking into account the fact that the ‘‘uptake" of magnesium by the cells is an insulin-regulated process, it will also be a process affected by insulin resistance,which may be responsible for the intracellular magnesium deficiency or aggravating an existing deficiency [62].

Glucose also appears to contribute to ionic cellular homeostasis independently of insulin. *In vitro* studies have shown direct suppression of magnesium resulting from hyperglycaemic states [62]. Since hyperglycaemia *per se* significantly contributes to insulin resistance

in diabetesmellitus and because the reduction of magnesium promotes vasoconstriction, it is hypothesized that these changes in magnesium induced by glucose are the cause of the vasoconstriction seen in chronic diabetes states [62,64-66].

Epidemiological studies have shown a high prevalence of hypomagnesemia in subjects with type II diabetes, especially in those with poor glycaemic control [62,64]. This cellular and extracellular magnesium depletion appears to be more severe in subjects with recent diagnosis [61].

Among the mechanisms in diabetes that may favour the depletion of magnesium found in these subjects are poor dietary intakes and increased urinary excretion of magnesium and calcium. Their absorption and retention is not altered in type II diabetes [62].

There is a negative correlation between serum magnesium concentration and microalbuminuria in middle-aged and elderly Chinese subjects with diabetes [61]. I was also found-that subjects with microalbuminuria or clinical proteinuria had a significant decrease inserum magnesium concentration compared to the non-microalbuminuria group [67].

The actual pathophysiological mechanism that explains this association is not well understood. Nevertheless, there are some mechanisms that potentially explain this relationship. One of these is the aforementioned link of hypomagnesemia to the insulin resistance state. Magnesium has a number of biological actions, including that of moderately potent calcium antagonist. In a situation of hypomagnesaemia, the relative intracellular increase of calcium may compromise adipocyte and skeletal muscle response to insulin with consequent development of insulin resistance [61]. Other studies report that insufficient serum levels of insulin or insulin resistance can affect the renal absorption of magnesium, decreasing it and contributing to the hypomagnesemia reported in diabetics. This vicious cycle may contribute to the increased risk of microalbuminuria in this population [53,61] (**Figure 4**).

Another proposed mechanism is related to oxidative stress, recognised as one of thecausative agents of microalbuminuria. Given the antioxidant properties of magnesium, Xu et al. suggest that this may be the mechanism that links hypomagnesemia and microalbuminuria [61].

On the other hand, there is evidence that serum magnesium concentration and the concentration of systemic inflammatory markers, whose role in the pathogenesis of microalbuminuria is crucial, have an inversely proportional relationship [61]. It is to be noted that several studies have shown the antioxidant and anti-inflammatory properties of magnesium [63].

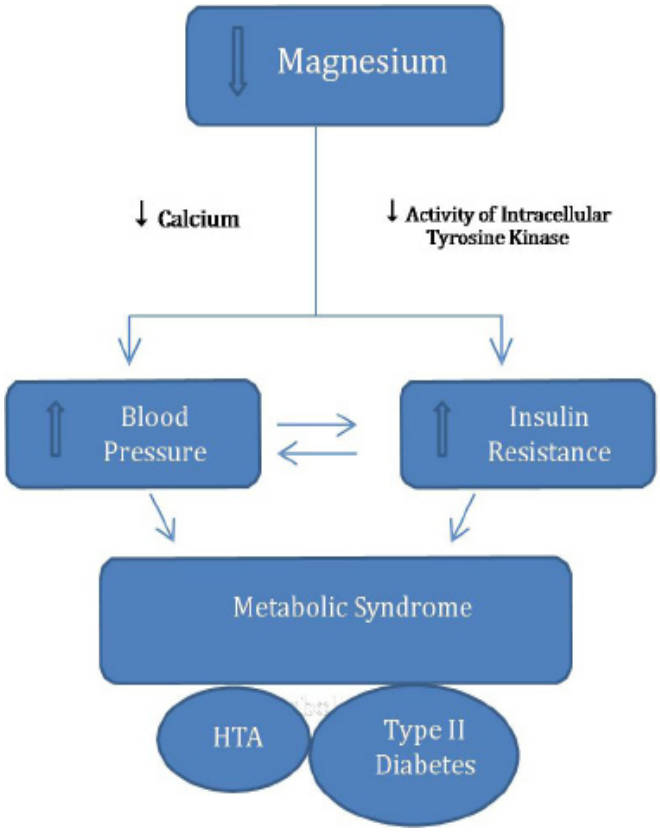

Figure 4: The contribution of magnesium to the metabolic syndrome

Magnesium deficiency may not only be secondary to type II diabetes but it may precede and cause insulin resistance *per se*, impaired glucose tolerance, and even type II diabetes [62,65-66]. Magnesium supplementation may improve the metabolic profile of diabetics but other studies did not show any benefit [62]. Once Magnesium is filtered by the kidney it should be administered with caution in patients with decreased glomerular filtration [63].

4. Conclusions

Diabetic nephropathy is the main cause of end-stage renal disease in the Western Countries. We should insist in the reduction of the incidence of diabetic nephropathy and in the delay of the progression of the renal insufficiency. The pathophysiology of the disease is complex and multifactorial. We must continue using the classical useful treatments such as the antagonists of renin-angiotens in system. However new players have been identified in the pathophysiology of the disease and new possibilities of intervention are possible.

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An eBook on Type 2 Diabetes

Chapter 4

Diabetic Nephropathy

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1. Introduction

Diabetic nephropathy is a chronic microvascular complication of diabetes and is the single most common cause of End Stage Renal Disease (ESRD) in Europe, Japan, and the United States, with diabetes accounting for 25% to 45% of all patients enrolled into ESRD programmes. The increased mortality in proteinuric diabetic patients is due not only from end-stagerenal disease (ESRD) but also from associated cardiovascular disease, with the latter being particularly common in type 2 diabetes patients. It often starts with microalbuminuria and progresses to macro albuminuria and sometimes to overt proteinuria with a decrease in glomerular filtration over a period of time which is slow and gradual (usually a number of years) and ultimately may require renal replacement therapy.

Diabetic nephropathy occurs in both type 1 (formerly called insulin-dependent or juvenile onset) and type 2 (formerly called non-insulin-dependent or adult onset) diabetes mellitus, as well as in other secondary forms of diabetes mellitus, for example after pancreatitis or pancreatectomy.

2. Epidemiology

The overall prevalence of microalbuminuria and macroalbuminuria is around 30% to 35% in both types of diabetes and this depends on a number of factors including the population studied. The highest prevalence, exceeding 50%, is found in Native Americans, followed by Asians, Mexican Americans, blacks, and European white patients [1]. Prevalence of albuminuria decreased but that of reduced glomerular filtration rate increased in patients with diabetes between 1988 and 2014 based on cross-sectional study of 6,251 patients ≥ 20 years old with diabetes in United States who completed surveys between 1988 and 2014 (NHANES III 1988-1994, NHANES 1999-2004, and NHANES 2005-2008). Age-adjusted incidence of diabetes-

related chronic kidney disease reported a decline from 299 to 197.7 per 100,000 persons with diabetes from 1990 to 2006. Potential reasons cited for this include:

- earlier detection and better management of kidney disease
- improved treatment and care of kidney disease
- better control of risk factors such as hypertension and glycaemic control
- large increase in new cases of diabetes (more patients who have not had diabetes long enough to develop CKD) [2].

- **Type 1 diabetes**- The epidemiology of diabetic nephropathy has been best studied in patients with type 1 disease as the time of clinical onset is usually known. Approximately 20 to 30 percent will have moderately increased albuminuria, formerly called "microalbuminuria," after a mean duration of diabetes of 15 years. Less than half of these patients will progress to overt nephropathy; moderately increased albuminuria may regress or remain stable in a substantial proportion, probably related to improved glycaemic and better blood pressure control with angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) [3,4].

- **Type 2 diabetes**- In Caucasians, the prevalence of progressive renal disease has generally been lower in type 2 diabetes than in type 1 disease. However, this observation may be a function of the usually later-onset disease and shorter-duration "exposure" in type 2 than type 1 diabetes. This may not, however, apply to all groups with type 2 diabetes, some of whom can have severe and progressive kidney disease and poor prognosis. Data seem to suggest that the renal risk is currently equivalent in the two types of diabetes. Evidence to support this hypothesis includes the observations that the time to proteinuria from the onset of diabetes and the time to ESRD from the onset of proteinuria was more or less similar in type 1 and type 2 diabetes [5].

2.1. Classification: Diabetic nephropathy can be classified according to a number of different subtypes:

2.1.1. Classification according to pathology (Renal pathology society classification)

- **Class I** - mild or nonspecific light microscopy changes and electron microscopy proven glomerular basement membrane thickening (isolated glomerular basement membrane thickening: basement membranes are greater than 430 nm in males older than age 9 years and 395 nm in females). There is no evidence of mesangial expansion, increased mesangial matrix, or global glomerulosclerosis involving >50 percent of glomeruli).

- **Class IIa** - mild mesangial expansion (in > 25% of observed mesangium)

- **Class IIb** - severe mesangial expansion (in > 25% of observed mesangium). A lesion is considered severe if areas of expansion larger than the mean area of a capillary lumen are present in >25 percent of the total mesangium.

- **Class III** - nodular sclerosis (at least 1 convincing Kimmelstiel-Wilson lesion) and<50 percent global glomerulosclerosis.

- **Class IV** - advanced diabetic glomerulosclerosis (global glomerular sclerosis in> 50% of glomeruli) with > 50 % glomerulosclerosis [6].

2.1.2. Classification according to severity of interstitial fibrosis and tubular lesion

- A score of 0 is assigned if the interstitium has no areas of interstitial fibrosis and tubular atrophy (IFTA); scores of 1, 2, or 3 are assigned if areas of IFTA <25, 25 to 50 or >50 percent, respectively.

- A score of 0 is assigned if no T lymphocytes or macrophage infiltrate is present. Scores of 1 or 2 are assigned if infiltrate is limited to the area surrounding atrophic tubules, or if infiltrate is not limited, respectively.

- Scores of 0, 1, or 2 are assigned if there is either no arteriolar hyalinosis, one arteriole, or more than one arteriole with hyalinosis is present. In addition, the most severely affected arteriole is assigned a score of 0, 1, or 2 if there is no intimal thickening, intimal thickening < thickness of media or intimal thickening > thickness of the media [7].

2.1.3. Classification according to glomerular filtration rate (GFR) and albuminuria category (Kidney Disease Improving Global Outcomes (KDIGO)

a] GFR categories

- G1 - GFR > 90 mL/minute/1.73 m2 (normal or high)

- G2 - GFR 60-89 mL/minute/1.73 m2 (mildly decreased compared to young adult level)

- G3a - GFR 45-59 mL/minute/1.73 m2 (mild-to-moderately decreased)

- G3b - GFR 30-44 mL/minute/1.73 m2 (moderate-to-severely decreased)

- G4 - GFR 15-29 mL/minute/1.73 m2 (severely decreased)

- G5 - GFR < 15 mL/minute/1.73 m2 (kidney failure)

b) Albuminuria categories

- A1 - albumin excretion rate (AER) < 30 mg/24 hours, albumin to creatinine ratio (ACR) < 30 mg/g (3 mg/mmol) (normal-to-mildly increased proteinuria)
- A2 - AER 30-300 mg/24 hours, ACR 30-300 mg/g (3-30 mg/mmol) (moderately increased proteinuria)
- A3 - AER > 300 mg/24 hours, ACR > 300 mg/g (30 mg/mmol) (severely increased proteinuria [including nephrotic syndrome]) [8].

2.2. Risk factors

There are a number of risk factors for the development of diabetic nephropathy. Some of the more likely factors are:

Genetic susceptibility: Genetic susceptibility is an important determinant of both the incidence and severity of diabetic nephropathy. The likelihood of developing diabetic nephropathy is increased in patients with a diabetic sibling or parent who has diabetic nephropathy; these observations have been made in both type 1 and type 2 diabetes patients [9].

Race: The incidence and severity of diabetic nephropathy is increased in the Afro-Caribbean population (3- to 6-fold increment compared to Caucasians), Mexican-Americans, and Pima Indians with type 2 diabetes. This observation in such genetically disparate populations suggests a primary role of socio-economic factors, such as diet as well as poor control of hyperglycaemia, hypertension, and obesity [10].

Age: The impact of age at onset of diabetes on the risk of developing nephropathy and end-stage renal disease is unclear. As an example, among patients with type 2 diabetes (but not type 1), increasing age, along with increasing duration of diabetes, has been associated with an increased risk for developing albuminuria in an Australian population [11].

Hypertension: Many prospective studies have noted an association between the development of diabetic nephropathy and higher systemic blood pressures [12].

Glomerular filtration rate: Those patients with glomerular hyperfiltration appear to be at increased risk for diabetic renal disease. This is particularly true for overt nephropathy if the initial GFR is above 150 mL/min; by comparison, lesser degrees of hyperfiltration may have a slower course of nephropathy development [13].

Poorly controlled diabetes it is a well-established fact that poor glycaemic control is associated with earlier onset of overt nephropathy development and this has been proven in several different studies [14].

Smoking: Smoking increases albuminuria and the risk of end-stage renal disease. It also has been shown to decrease survival once patients are on dialysis [15].

Obesity - A high body mass index (BMI) has been associated with an increased risk of chronic kidney disease among patients with diabetes. Diet and weight loss has been shown to reduce albuminuria and improve kidney function in such patients [16].

Oral Contraceptive Pill (OCP) - There has been a suggested link between oral contraceptive use and the risk of development of diabetic nephropathy in one report [17].

Hypercholesterolemia - Poorly controlled cholesterol level is also associated with adverse prognosis related to diabetic kidney disease [18].

Persistently increased albuminuria: Severely increased albuminuria, formerly called "macro-albuminuria," is strongly associated with and predictsfuture development of severe nephropathy with decreased eGFR [19].

Acute kidney injury: Acute kidney injury is associated with increased future risk of developing advanced chronic kidney disease in patients with diabetes [20].

Vitamin D deficiency: This may be associated with increased risk of diabetic nephropathy. This is based on a cross-sectional study of 1,216 adults ≥ 20 years old with diabetes who were evaluated for vitamin D deficiency and insufficiency and occurrence of diabetic nephropathy - nephropathy was seen in 30.7% of those who were Vitamin D deficient or insufficient [21].

3. Relationship between Retinopathy/Neuropathy and Diabetic Nephropathy

Patients with nephropathy and type 1 diabetes almost always have other signs of diabetic microvascular disease, such as retinopathy and neuropathy. Retinopathy is easy to detect clinically, and typically precedes the onset of overt nephropathy in these patients. The converse is not true. Type 2 diabetes patients with marked proteinuria and retinopathy most likely have diabetic nephropathy, while those without retinopathy tend to have a high frequency of nondiabetic kidney disease. Blindness due to severe proliferative retinopathy or maculopathy is approximately five times more frequent in types 1 and 2 diabetic patients with nephropathy than in normoalbuminuric patients. Macroangiopathies (e.g., stroke, carotid artery stenosis, coronary heart disease, peripheral vascular disease) are two to five times more common in patients with diabetic nephropathy and, as stated earlier, macroangiopathy is the major cause of mortality rather than ESKD for patients with diabetic nephropathy [22]. Peripheral neuropathy is present in almost all patients with advanced nephropathy. Foot ulcers with sepsis leading to amputation occur frequently (>25% of cases), probably due to a combination of neural and arterial diseases. Autonomic neuropathy may be asymptomatic and manifest simply as abnor-

mal cardiovascular reflexes, or it may result in debilitating symptoms. Almost all patients with nephropathy have grossly abnormal results on autonomic function tests [23].

4. Nondiabetic Kidney Disease

Proteinuria and/or haematuria in diabetes mellitus is occasionally due to a glomerular disease other than diabetic nephropathy. Examples include membranous nephropathy, minimal change disease, IgA nephropathy, focal segmental glomerulosclerosis, Henoch-Schönlein purpura (IgA vasculitis), thin basement membrane disease, proliferative glomerulonephritis, collapsing glomerulopathy, and pauci-immune crescentic glomerulonephritis. With regards to membranous nephropathy, porcine insulin has been implicated to be an inciting antigen in some patients.

When to suspect non-diabetic kidney disease?

1. Onset of proteinuria less than five years from the documented onset of type 1 diabetes

2. Acute onset of renal disease.

3. The presence of an active urine sediment containing red cells (particularly acanthocytes) and cellular casts. However, haematuria and red cell casts can also be occasionally seen in diabetic nephropathy.

4. Absence of retinopathy or neuropathy [24].

5. Pathogenesis

There are a number of mechanisms by which hyperglycemia triggers the basic pathogenic changes in diabetic nephropathy. Listed below are some common immune triggers and markers:

Hyperglycaemia may directly induce mesangial expansion and injury, perhaps in part via increased matrix production or glycation of matrix proteins. In vitro studies have demonstrated that hyperglycaemia stimulates mesangial cell matrix production and mesangial cell apoptosis. Glomerulosclerosis may result from intra glomerular hypertension induced by renal vasodilatation, or from ischemic injury induced by hyaline narrowing of the vessels supplying the glomeruli. The role of glomerular hypertension and hyperfiltration in diabetic nephropathy is reinforced by the apparent benefits of blockade of the renin-angiotensin system. Antagonizing the profibrotic effects of angiotensin II may also be a significant factor in benefits observed with these agents [25].

Glycation of tissue proteins also may contribute to the development of diabetic nephropathy and other microvascular complications. In chronic hyperglycemia, some of the excess

glucose combines with free amino acids on circulating or tissue proteins. This non-enzymatic process initially forms reversible early glycation products and, later, irreversible advanced glycation end products (AGEs). Circulating AGE levels are increased in diabetes, particularly those with renal insufficiency, since AGEs are normally excreted in the urine. The net effect is tissue accumulation of AGEs, in part by crosslinking with collagen, which can contribute to the associated renal and microvascular complications. Other proposed mechanisms by which hyperglycaemia might promote development of diabetic nephropathy include activation of protein kinase C, and upregulation of heparanase expression.

Activation of cytokines, profibrotic elements, inflammation, and vascular growth factors (vascular endothelial growth factor, VEGF) may be involved in the matrix accumulation in diabetic nephropathy. A potentially pathogenic role for VEGF in diabetic nephropathy is supported by the observation that VEGF blockade improves albuminuria in experimental models of diabetic nephropathy [26]. Hyperglycaemia also increases the expression of transforming growth factor-beta (TGF-beta) in the glomeruli and diabetes is associated with decreased expression of renal bone morphogenic protein-7 (BMP-7), which appears to counter the profibrogenic actions of TGF-beta [27]. It has been shown that the administration of hepatocyte growth factor, which specifically blocks the profibrotic actions of TGF-beta, ameliorates diabetic nephropathy in mice. Renal expression of nephrin may be impaired in diabetic nephropathy. When compared with nondiabetic patients with minimal change nephropathy and controls, patients with diabetic nephropathy had markedly lower renal nephrin expression [28].

Defects in podocyte-specific insulin signalling may contribute to diabetic nephropathy. Mouse models have been generated in which the gene encoding the insulin receptor is deleted in a podocyte-specific manner.

6. Comparison of Nephropathy between Type 1 and Type 2 diabetes:

- Renal pathology and structural-functional relationships has been less well studied in type 2 diabetes patients, despite more than 80% of diabetes patients with ESRD have type 2 diabetes.

- Proteinuric white Danish patients with type 2 diabetes were reported to have structural changes similar to those of proteinuric patients with type 1 diabetes, and the severity of these changes was strongly correlated with the subsequent rate of decline of GFR; however, the study also noted that some proteinuric patients with type 2 diabetes had little or no evidence of diabetic glomerulopathy.

- A study of 52 type 2 diabetic patients from Northern Italy who underwent biopsy for clinical indications described greater heterogeneity in renal structure, with one third having

nondiabetic renal diseases.

• In a Danish study, 75% of unselected proteinuric type 2 diabetic patients had histological evidence of diabetic nephropathy but 25% had a variety of nondiabetic glomerulopathies, including minimal lesions, glomerulonephritis, mixed diabetic and glomerulonephritis changes, and chronic glomerulonephritis [29]. Marked heterogeneity in renal histopathological structure is present in type 2 diabetic patients with increased proteinuria.

• Only a minority of patients have histopathologic patterns resembling those typically present in patients with type 1 diabetes; the typical pattern is 30% of patients with microalbuminuria and 50% of those with proteinuria. The remainder have minimal renal abnormalities or tubulo-interstitial, vascular, and global glomerulosclerotic changes, which are disproportionally severe relative to the diabetic glomerulopathy lesions (atypical pattern, about 40% of patients with microalbuminuria and proteinuria).

• In type 2 diabetes, a reduced GFR in the presence of a normal albumin excretion rate is a common finding. Ekinci and colleagues have recently described that among normoalbuminuric type 2 diabetic patients with impaired renal function, only a subset had typical diabetic glomerulopathy, whereas the remaining patients had predominantly tubulo-interstitial and vascular changes [30].

• Most studies dealing with the natural history of diabetic nephropathy have demonstrated a relentless, often linear rate of decline in GFR. Importantly, this rate of decline is highly variable across individuals, ranging from 2 to 20 mL/min/yr, with a mean of about 12 mL/min/yr. Type 2 diabetes patients with nephropathy display the same degree of loss in filtration function and variability of GFR.

7. Other Renal Pathology found in Patients with Diabetes

• Minimal change nephrotic syndrome and membranous nephropathy occur with greater frequency in patients with type 1 diabetes than in nondiabetic persons.

• Fewer than 1% of patients with type 1 diabetes for 10 years or longer and fewer than 4% of those with proteinuria and long duration of diabetes will be found to have conditions other than, or in addition to, diabetic nephropathy.

• Proteinuric type 2 diabetic patients without retinopathy may have a high incidence of atypical renal biopsy findings or other diseases. Proteinuric patients with type 1 diabetes of less than 10 years' duration and type 2 diabetic patients without retinopathy should be thoroughly evaluated for other renal diseases, and a renal biopsy is strongly considered.

- **Other causes of nodular glomerulosclerosis**:

  - Dysproteinemias such as amyloidosis and monoclonal immunoglobulin deposition diseases (MIDD), mostly kappa light chain deposition disease.

  - Organized glomerular deposition diseases, fibrillary and immunotactoid glomerulonephritis, fibronectin glomerulopathy, and collagen III glomerulopathy.

  - Chronic hypoxic or ischemic conditions, such as cyanotic congenital heart disease, Takayasu's arteritis with renal artery stenosis, or cystic fibrosis

  - Chronic membrano-proliferative glomerulonephritis (type I)

  - Idiopathic nodular glomerulosclerosis, which is frequently associated with smoking, hypertension and metabolic syndrome, but without overt diabetes mellitus [31].

8. Reversibility of Diabetic Nephropathy Lesion

Mesangial expansion present after 7 months of diabetes was reversed within 2 months after normoglycemia was induced by islet transplantation in rats with streptozotocin-induced diabetes. But it was disappointing to note that no improvement in diabetic nephropathy lesions in their native kidneys was found after 5 years of normoglycemia following successful pancreatic transplantation in type 1 patients with a diabetes duration of approximately 20 years. After 10 years of normoglycemia, however, these same patients showed marked reversal of diabetic glomerulopathy lesions. Thus, GBM and TBM width were reduced at 10 years compared with the baseline and 5-year values, with several patients having values at 10 years that had returned to the normal range; primarily due to a marked decrease in mesangial matrix fractional volume. Remarkable glomerular architectural remodelling was seen by light microscopy, including the complete disappearance of Kimmelstiel-Wilson nodular lesions. The reason for the long delay in this reversal process is not understood but could include epigenetic memory of the diabetic state, the slow process of replacement of glycated by nonglycated ECM, or other as yet undetermined processes. More recently, remodelling and healing in the tubulo-interstitium has also been demonstrated in these same patients. These studies have demonstrated reduction in total cortical interstitial collagen and underscore the remarkable potential for healing of kidney tissue that has been damaged by long-standing diabetes. Blockade of the renin angiotensin aldosterone system (RAAS) for 5 years did not lead to regression or slowing of the progression of diabetic glomerulopathy lesions in young patients with type 1 diabetes and normoalbuminuria. Whether healing can be induced by treatments other than cure of the diabetic state is currently unknown [32].

9. Diabetic Nephropathy and Hypertension

• Several studies have demonstrated blood pressure elevation in children and adults with type 1 diabetes and microalbuminuria.

• The prevalence of arterial hypertension (according to Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [33] criterion of ≥140/90 mm Hg) in adult patients with type 1 diabetes increases with urine albumin level, and prevalence rates reported are 42%, 52%, and 79% in those with normoalbuminuria, microalbuminuria, and macroalbuminuria, respectively.

• The prevalence of hypertension in those with type 2 diabetes (mean age, 60 years) was reported to be higher in comparison —71%, 90%, and 93% in the normoalbuminuria, microalbuminuria, and macroalbuminuric groups, respectively.

• A genetic predisposition to hypertension in type 1 diabetes patients who develop diabetic nephropathy has been suggested, but other studies have not confirmed this. The original finding was confirmed by using 24-hour blood pressure monitoring in a large group of parents of type 1 diabetic patients, with and without diabetic nephropathy [33].

• The cumulative incidence of hypertension was found to be higher among parents of proteinuric patients, with a shift toward a younger age at the onset of hypertension in this parental group. However, the difference in prevalence of parental hypertension was not evident when office blood pressure measurements were used.

• Several studies have reported that sodium and water retention play a dominant role in the initiation and maintenance of systemic hypertension in patients with microalbuminuria and diabetic nephropathy, whereas the contribution of the RAAS is somewhat smaller [33].

10. Albuminuria

• A preclinical phase of diabetic nephropathy consisting of a normoalbuminuric and microalbuminuric stage and a clinical phase characterized by albuminuria has been well documented in both types 1 and 2 diabetic patients.

10.1. Normoalbuminuria

• Approximately one third of type 1 diabetic patients will have a GFR above the upper normal range for age-matched healthy nondiabetic subjects. The degree of hyperfiltration is less in type 2 diabetic patients, and hyperfiltration is even reported to be lacking in some studies [34].

• The GFR elevation is particularly pronounced in patients with newly diagnosed

diabetes and during other intervals with poor metabolic control. Intensified insulin treatment and control to near-normal blood glucose levels reduces the GFR toward normal levels after a period of days to weeks in both types 1 and 2 diabetes patients

- Four factors seem to regulate GFR. Firstly, the glomerular plasma flow influences the mean ultrafiltration pressure and there by GFR. Enhanced renal plasma flow has been demonstrated in both types 1 and 2 diabetic patients with elevated GFR. Secondly, GFR is also regulated by the systemic oncotic pressure, which is reported to be normal in diabetes as calculated from plasma protein concentrations. The third determinant of GFR is the glomerular trans-capillary hydraulic pressure difference, which cannot be measured in humans. However, the demonstrated increase in filtration fraction is compatible with an enhanced transglomerular hydraulic pressure difference. The last determinant of GFR is the glomerular ultrafiltration coefficient, [Kf], which is determined by the product of the hydraulic conductance of the glomerular capillary and the glomerular capillary surface area available for filtration. Total glomerular capillary surface area is clearly increased at the onset of human diabetes.

- Longitudinal studies have suggested that hyperfiltration is a risk factor for subsequent increase in urinary albumin excretion and development of diabetic nephropathy in type 1 diabetic patients, but conflicting results have also been reported.

- The prognostic significance of hyperfiltration in type 2 diabetic patients is still debated. Six prospective cohort studies following normoalbuminuric types 1 and 2 diabetic patients for 4 to 10 years revealed that slight elevation of urinary albumin excretion, which remained in the normal range and poor glycemic control, hyperfiltration, elevated arterial blood pressure, retinopathy, and smoking contribute to the development of persistent microalbuminuria and overt diabetic nephropathy [35].

- For reasons that are not well understood, the degree of albuminuria is not necessarily linked to disease progression in patients with diabetic nephropathy in both type 1 or type 2 diabetes. This was illustrated in a report of 79 patients with type 1 diabetes from the Joslin Kidney Study who were followed for a mean of 12 years after the onset of moderately increased albuminuria [36].

- Recently, an elevated serum uric acid level was found to be a predictor of the development of diabetic nephropathy in type 1 diabetic patients, and a multicentre study has been initiated to study whether lowering uric acid in patients with early diabetic nephropathy preserves renal function.

10.2. Moderately increased albuminuria

- Recently, for CKD in general, it has been suggested to use the term moderately *in-*

creased albuminuria [37] *instead* of microalbuminuria. In addition to hyperglycemia, many other factors can induce microalbuminuria in diabetic patients, such as hypertension, obesity, heavy exercise, various acute or chronic illnesses, and cardiac failure.

• The demonstration that microalbuminuria diminishes promptly with acute reduction in arterial blood pressure argues that reversible hemodynamic factors play an important role in the pathogenesis of microalbuminuria. Imanishi and colleagues have demonstrated that glomerular hypertension is present in type 2 diabetic patients with early nephropathy and is closely correlated with increased urinary albumin excretion. In addition, increased pressure has been demonstrated in the nail fold capillaries of microalbuminuric type 1 diabetic patients.

• GFR, measured using the single-injection, chromium-51–radiolabelled ethylenediaminetetraacetic acid (51Cr-EDTA) plasma clearance method or renal clearance of inulin is normal or slightly elevated in type 1 diabetic patients with microalbuminuria. Prospective studies have demonstrated that GFR remains stable at normal or supranormal levels for at least 5 years if clinical nephropathy does not develop. Nephromegaly is still present and is even more pronounced in micro-albuminuric than in normo-albuminuric type 1 diabetic patients. In microalbuminuric type 2 patients, GFR declines at rates of about 3 to 4 mL/min/yr.

11. Haematuria

• The urine sediment in diabetic nephropathy is usually bland and haematuria is unusual in diabetes. However, microscopic haematuria can occur as it can in any form of glomerular disease, including disorders such as membranous nephropathy that are not associated with glomerulonephritis. This is an important issue in diabetic nephropathy since nondiabetic renal disease, either alone or with diabetic nephropathy, is occasionally seen in patients with diabetes.

• Red blood cell casts have also been described in patients with diabetic nephropathy. The clinical significance of this finding was evaluated in a study of eight patients with haematuria, red blood cell casts, and known diabetes for 6 to 18 years. Renal biopsy, including immunofluorescence and electron microscopy, revealed glomerulonephritis in three (post-infectious in two and IgA nephropathy in one). The other five patients had only diabetic nephropathy.

11.1. Making the diagnosis

One needs to consider a diagnosis of diabetic kidney disease patients with diabetes with any of the following:

Albuminuria of > 300 mg/24 hours or albumin to creatinine ratio > 300 mg/g (also called macroalbuminuria) [38]. It has been suggested that adjusting the urinary albumin concentration for urinary creatinine concentration may not only correct for diuresis, but elevated

ratios may reflect two independent risk factors-elevated albumin excretion, reflecting renal and vascular damage, and reduced creatinine excretion, associated with reduced muscle mass.The excretion of albumin in the urine is determined by the amount filtered across the glomerular capillary barrier and the amount reabsorbed by the tubular cells. A normal urinary β2 -microglobulin excretion rate in microalbuminuria suggests that albumin is derived from enhanced glomerular leakage rather than from reduced tubular reabsorption of protein.

Albuminuria of 30-300 mg/24 hours or albumin to creatinine ratio 30-300 mg/g (also called microalbuminuria) plus retinopathy.

Microalbuminuria plus type 1 diabetes of ≥ 10 years duration- the daily variation in urinary AER is high, 30% to 50%. Consequently, more than one urine sample is needed to determine whether an individual patient has persistent microalbuminuria. Urinary albumin excretion in the micro-albuminuric range (30 to 300 mg/24 hr) in at least two of three consecutive nonketotic sterile urine samples is the generally accepted definition of persistent microalbuminuria. For convenience, it has been recommended to use early morning spot urine samples for screening and monitoring.

Albuminuria based on elevated albumin to creatinine ratio should be confirmed with additional 2 first-voided urine specimens during subsequent 3-6 months. Determination of the IgG/IgG 4 ratio suggests that loss of glomerular charge selectivity precedes or accompanies the formation of new glomerular macromolecular pathways in the development of diabetic nephropathy. Reduction in the negatively charged moieties of the glomerular capillary wall, particularly sialic acid and heparan sulphate, has been suggested, but not all studies have confirmed these findings [39].

Consider nondiabetic causes of chronic kidney disease if any of the following are present:

- absence of diabetic retinopathy
- low or rapidly decreasing glomerular filtration rate
- rapidly increasing proteinuria or nephrotic syndrome
- refractory hypertension
- active urinary sediment
- signs or symptoms of other systemic disease
- > 30% reduction in glomerular filtration rate within 2-3 months after starting angiotensin-converting enzyme inhibitor or angiotensin receptor blocker therapy

Causes of transient increases in albuminuria include:

- fever
- high-salt diet
- vigorous exercise in previous 24 hours
- infection
- dehydration
- heart failure

12. Investigations

Annual screening for diabetic kidney disease is recommended startingat diagnosis with type 2 diabetes and ≥ 5 years after diagnosis with type 1 diabetes. Screening should includemeasurement of urinary albumin to creatinine ratio (ACR) in spot urine sample and measurement of serum creatinine and estimation of estimated glomerular filtration rate (eGFR). Additional testing should be done if abnormal urine albumin excretion is present persistently: this may include confirmation first by 2 additional urine specimen testing for ACRwithin 1-2 months, tests to rule out nondiabetic causes of kidney disease if suspicion exists and kidney biopsy if uncertainty exists about the exact diagnosis.

13. Monitoring Blood Glucose Control in Patients with Chronic Kidney Disease

National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) suggests using HbA1c to measure blood sugar control in patients with CKD and diabetes. However, HbA1C may not be reflective of glucose control in people with CKD who have reduced red cell life span, and thus should be interpreted with caution; reviewing blood sugar daily logs may be more reliable. Albumin corrected fructosamine was reported to reliably indicate glycaemic control in 30 patients with diabetes and CKD stages 3-4 but HbA1c underestimated glycaemic control. Glycated albumin is reported to more accurately reflect glycaemic control compared to fructosamine and HbA1c in 25 patients with diabetes and CKD stage 4-5. Glycated albumin was also found to better estimate glycaemic control than HbA1c detected in a study in 25 patients with diabetes on haemodialysis. HbA1c and albumin-corrected fructosaminehas been reported to reliably indicate glycaemic control in patients with diabetes on peritoneal dialysis.

14. Kidney Biopsy and Pathology

Kidney biopsy is not often required but may be occasionally undertaken to definitely

diagnose diabetic glomerulopathy (possibility exists of serious diabetic glomerular lesions in patients with normoalbuminuria and normal glomerular filtration rate) in patients with a suspicion of non-diabetic kidney pathology. Typical findings in patients with type 1 diabetes and macroalbuminuriamay include increased mesangial volume and glomerular basement membrane (GBM) thickness. Tubulo-interstitial pathology findings in patients with type 1 diabetes and microalbuminuria are generally less severe. In patients with type 2 diabetes and microalbuminuria, about 40% show changes similar to type 1 diabetes, 30% show tubulo-interstitial, vascular, and/or glomerulosclerotic lesions unrelated to classic diabetic glomerulopathy and about 30% have normal or near normal biopsy results.

15. Treatment

15.1. Protein restriction

American Diabetes Association (ADA) recommendations [40] on protein intake in patients with diabetes and diabetic kidney disease recommends normal daily protein allowance (0.8 g/kg/day) for patients with non-dialysis dependent diabetic nephropathy and a higher protein intake for patients on dialysis. National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) recommends target dietary protein intake 0.8 g/kg/day for people with diabetes and chronic kidney disease stages 1-4. Protein-restricted diet may slightly slow progression of diabetic nephropathy and might reduce risk of end-stage kidney disease or death. Low-protein diet may reduce proteinuria and HbA1c but may not improve glomerular filtration rate. Reducing animal protein in diet may improve cardiovascular risk measures and might improve markers of kidney function.

A low-carbohydrate, low-iron diet enriched with polyphenols may delay progression of nephropathy and reduce composite outcome of kidney replacement therapy or death compared to conventional protein-restricted diet in patients with type 2 diabetes.

15.2. Salt intake and proteinuria

A high salt intake has been shown to blunt the antiproteinuric effects of angiotensin inhibitors in patients with nondiabetic kidney disease. Salt restriction and/or diuretics enhance the effect of renin-angiotensin blockade on proteinuria in these patients. Thus, patients on ACE inhibitors or ARBs who do not have sufficient reduction in proteinuria despite appropriate blood pressure goals should be instructed to take a low-sodium diet. An assessment of baseline sodium intake can be undertaken by obtaining a 24-hour urine for sodium and creatinine. This can be repeated after several months on the low-sodium diet to determine the actual sodium intake if there is insufficient reduction in proteinuria. Salt restriction to ≤70 meq/day has been found to enhance the antiproteinuric effects of ARB in patients with type 2 diabetes. However, this degree of restriction may be difficult to achieve and maintain, and it is recom-

mended restricting sodium intake to approximately ≤100 meq/day. If a low-sodium diet is not possible, administration of a diuretic partially corrects the loss of antiproteinuric effect due to a high sodium intake [3,40].

15.3. Glycaemic control

HbA1c target < 7% (53mmol/mol) has been recommended for most nonpregnant adults with type 1 or type 2 diabetes [41], but goals should be individualized. HbA1c about 7% (53mmol/mol) is recommended to prevent or delay progression of microvascular complications of diabetes including diabetic nephropathy. One may need to consider more stringent target (such as < 6.5% or 48mmol/mol) in selected patients (early in disease course) if achievable without significant hypoglycaemia or other adverse effects. Less stringent target (such as < 8% or 64mmol/mol) may be appropriate for patients with risk for hypoglycemia, comorbidities, or limited life expectancy.

In the Diabetes Control and Complications Trial (DCCT) [42], intensive therapy reduced the occurrence of microalbuminuria by 39% (95% CI, 0.21 to 0.52), and that of albuminuria by 54% (95% CI, 0.19 to 0.74) when the primary and secondary prevention cohorts were combined for analysis.

With further follow-up of DCCT patients in the Epidemiology of Diabetes Interventions and Complications (EDIC) study [42], it was demonstrated that the reduction in the development of microalbuminuria and albuminuria translated into a 50% reduced risk (95% CI, 0.18 to 0.69; P = 0.006) of development of impaired renal function (eGFR < 60).

In Japanese type 2 diabetic patients, a beneficial impact of strict glycaemic control on the progression of normoalbuminuria to microalbuminuria and macroalbuminuria was also demonstrated in a small study with a design similar to that of the DCCT.

Results of this study have been confirmed and extended by data from the UK Prospective Diabetes Study (UKPDS) [43] documenting a progressively beneficial effect of intensive metabolic control on the development of microalbuminuria and overt proteinuria, and a 10-year post study follow-up demonstrated a long-lasting beneficial effect. This beneficial effect was confirmed in the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified-Release Controlled Evaluation (ADVANCE) study [44], in which 11,140 patients with type 2 diabetes were followed for a median of 5 years and a 21% reduction in the development of nephropathy (95% CI, 0.07 to 0.34) was seen in patients randomly assigned to strict glycemic control. The same trend was seen in the smaller Veterans Affair Diabetes Trial [45], but the values did not reach statistical significance.

15.4. Insulin

Intensive insulin therapy may delay onset of and slow progression of microalbuminuria and albuminuria (reduced risk of increased serum creatinine, albuminuria, and hypertension may continue 7-8 years after intensive therapy). This is based on evidence from the Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Intervention and Complications (EDIC) observation findings of long-term benefit of initial intensive control referred to as metabolic memory. It is advisable to monitor blood glucose levels closely in patients with worsening kidney function and adjust dose of appropriate medications as needed to avoid hypoglycaemia.

15.5. Benefits of intensive insulin therapy

It can partially reverse the glomerular hypertrophy and hyperfiltration (both in the basal state and after a protein load) that are thought to be important risk factors for glomerular injury. It can delay the development of elevated albumin excretion. Intensive therapy to near-normal glycaemia reduces the onset or progression of diabetic nephropathy for years after less intensive therapy. It can stabilize or decrease protein excretion in patients with increased albumin excretion, although this effect may not be apparent until relative normoglycemia has been maintained for about two years. Furthermore, the restoration of eu-glycaemia with pancreas transplantation in patients with type 1 diabetes prevents recurrent nephropathy in a renal allograft.It can slow the progression of glomerular filtration rate (GFR) decline.Studies suggest that strict glycaemic control may also slow the rate of progressive renal injury even after overt dipstick-positive proteinuria has developed.

15.6. Metformin

Metformin is contraindicated in renal failure when eGFR is less than 30ml/min (dose needs to be reduced to 1gm/day when eGFR is between 30-45ml/min). Risk of lactic acidosis is present but probably over-exaggerated [42].

15.7. Sulfonylureas

First-generation sulfonylureas (chlorpropamide) have been associated with prolonged and severe hypoglycemia in patients with poor kidney function. They may also aggravate heart failure or fluid retention (antidiuretic effect) and may cause syndrome of inappropriate antidiuretic hormone.

Second generation sulfonylurea as like gliclazide and glipizide elimination half-life is generally not affected by renal dysfunction. They are the sulfonylureas of choice in patients with advanced kidney disease dosage but adjustment may be necessary to avoid hypoglycaemia. With glyburide, there is increased risk of hypoglycemia in patients with kidney failure

and patients with advanced chronic kidney disease due to increased half-life.

Third generation sulphonylureas like glimeperide needs dose reduction in patients with decreased GFR and prolonged hypoglycemia has been reported in patients with renal dysfunction.

15.8. Thiazolidinediones (glitazones)

Pioglitazone does not need dose adjustment needed in patients with poor renal reserve and can be used even in patients with stage 5 CKD. In the setting of renal failure, caution is needed against heart failure and fluid retention.

15.9. Meglitinides

Repaglinide half-life is increased with kidney dysfunction and in patients on dialysis. No dose reduction is needed with chronic kidney disease, caution is advised with kidney impairment. Nateglinide has been associated with hypoglycemia in patients with low GFR and should be used cautiously in patients with kidney disease.

15.10. Dipeptidyl peptidase IV (DPP-4) inhibitors (Gliptins)

Gliptins are safe to be used in renal impairment. Whereas no dose reduction is required with linagliptin (mostly excreted in bile)- dosage adjustment is advocated with most other gliptins like sitagliptin, saxagliptin, alogliptin and vildagliptin as all these are predominantly renally excreted [46].

15.11. Glucagon-like peptide-1 (GLP-1) receptor agonists

Exenatide should be avoided if creatinine clearance < 30 mL/minute. Liraglutide can be used up to an eGFR of 30ml/min but caution is required when initiating or escalating dose in patients with renal failure. Once weekly dulaglutide has also got license to be used up to an eGFR of 30ml/min [47].

15.12. Sodium-glucose cotransporter 2 (SGLT2) inhibitors

SGLT2 inhibitors as a class in patients with renal impairment is not recommended when eGFR is less than 60 ml/min although recent trial EMPA-REG has shown some benefit in proteinuria reduction and slowing progression in CKD when used in patients with eGFR < 60ml/min [48].

16. Blood Pressure Control

Blood pressure targets in patients with diabetes varies, and can range from < 130/80 mm Hg to < 140/90 mm Hg. However, most of these BP recommendations are not specific to

patients with diabetes and chronic kidney disease (CKD). National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) guidelines recommend target blood pressure < 130/80 mm Hg for patients with diabetes and chronic kidney disease. Kidney Disease: Improving Global Outcomes (KDIGO) guideline for adults with chronic kidney disease and diabetes recommend target blood pressure ≤ 140/90 mm Hg if urine albumin excretion < 30 mg/24 hours and ≤ 130/80 mm Hg if urine albumin excretion > 30 mg/24 hours. American Diabetes Association (ADA) recommends systolic blood pressure < 140 mm Hg and diastolic blood pressure < 90 mm Hg to reduce risk or slow progression of diabetic kidney disease. Eighth Joint National Committee (JNC 8) recommends target blood pressure < 140/90 mm Hg for patients with diabetes (JNC8 Expert opinion) and for patients with chronic kidney disease but did not make recommendation specific to combination of diabetes and chronic kidney disease [49].

- Angiotens in-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) should be used in patients with diabetes if
- urinary albumin excretion ≥ 300 mg/day
- consider use if urinary albumin excretion 30-299 mg/day
- urine albumin to creatinine ratio ≥ 30 mg/g
- chronic kidney disease and hypertension.
- Monitor serum creatinine and potassium levels periodically for worsening of serum creatinine and hyperkalaemia if using ACE inhibitors, ARBs, or diuretics. Consider using diuretic, calcium channel blocker, or beta blockers if additional therapy needed to control blood pressure or if patient unable to tolerate ACE inhibitors or ARBs
- In pregnant women with diabetes and chronic kidney disease (NKF KDOQI), ACE inhibitors and ARBs before pregnancy may improve foetal and maternal outcomes, but discontinue as soon as a menstrual period is missed or after a positive pregnancy test. Use insulin to control hyperglycaemia if pharmacologic therapy needed.
- Canadian Society of Nephrology [50] recommends against prescribing angiotensin converting enzyme (ACE) inhibitors in combination with angiotensin II receptor blockers (ARBs) for the treatment of hypertension, diabetic nephropathy and heart failure.

17. Angiotensin-Converting Enzyme (ACE) Inhibitors and ARB

The summary of all the trial evidence regarding the benefits of ACE inhibitor and ARBs are as follows:

• The **DIRECT study** did not show any significant effect on the incidence of microalbuminuria [51].

• The **BENEDICT study** has demonstrated that use of an ACE inhibitor, alone or in combination with a calcium channel blocker, decreases the incidence of microalbuminuria in hypertensive type 2 diabetic patients with normoalbuminuria [52]. The effect of the calcium channel block verapamil alone was similar to that of placebo.

• In the **ADVANCE study**, which included type 2 diabetic patients with or without hypertension, the fixed combination of perindopril and the diuretic indapamide reduced blood pressureand new-onset microalbuminuria by 21% [44].

• The Randomized Olmesartan and Diabetes Microalbuminuria Prevention **(ROADMAP) study** tested whether the angiotensin II receptor blocker olmesartan would reduce development of microalbuminuria in 4447 mostly hypertensive type 2 diabetic patients with normoalbuminuria. Overall, there were slightly fewer cardiovascular events with olmesartan but more fatal events, although numbers were very small[53].

• In the **ONTARGET study**, 25,620 patients with atherosclerotic disease or diabetes (38% with diabetes) who had end-organ damage were randomly assigned to treatment with an ACEI, angiotensin receptor blocker (ARB), or both and were followed for a median of 56 months. Although the combination treatment reduced the increase in urinary AER, the number of events for the composite primary outcome or doubling of the serum creatinine level, need for dialysis, or death was similar for telmisartan (N = 1147 [13.4%]) and ramipril (N = 1150 [13.5%]; HR, 1.00; 95% CI, 0.92 to 1.09) but was increased with combination therapy (N = 1233 [14.5%]; HR, 1.09; 95% CI, 1.01 to 1.18; P = 0.037). It is important to stress that the ONTARGET study did not include significant numbers of patients with overt diabetic nephropathy. Therefore, the therapeutic risk or benefit for RAAS combination therapy in patients with diabetic nephropathy could not be addressed by this study [54].

• Patients intolerant to ACE inhibition (n = 5927), but otherwise similar at baseline to patients enrolled in the ONTARGET study, were randomly assigned to receive a placebo or ARB in the **TRANSCEND study**. Albuminuria increased less in patients receiving the ARB than in those receiving placebo (32% [95% CI, 0.23 to 0.41] vs. 63% [95% CI, 0.52 to 0.76]). Very few patients (<2%) reached the prespecified renal end points, which were identical to those of the ONTARGET study, and no difference was seen between treatment groups with regard to these end points [55].

• Parving and co-workers have evaluated the renoprotective effect of the angiotensin II receptor antagonist irbesartan in hypertensive patients with type 2 diabetes and microalbuminuria in the **IRMA 2 trial**. Remission to normoalbuminuria was more common in the irbe-

sartan-treated patients than in those treated with placebo [56].

- The importance of this finding is a slower decrease in GFR, as also demonstrated in the **STENO-2 study** [57].

- The antiproteinuric effect of ACE inhibition in patients with diabetic nephropathy varies considerably. Individual differences in the RAAS may influence this variation. Therefore, the potential role of an ID polymorphism of the ACE gene on this early antiproteinuric responsiveness was tested in an observational follow-up study of young type 1 diabetic patients with hypertension and diabetic nephropathy. The study found that type 1 diabetes patients with the homozygous II genotype were particularly likely to benefit from commonly advocated renoprotective treatment [58].

- The **EUCLID Study** demonstrated that urinary AER during lisinopril treatment was 57% lower in the II group, 19% lower in the ID group, and 19% higher in the DD group compared with the placebo group. Furthermore, the polymorphism of the ACE gene predicts therapeutic efficacy of ACEIs against the progression of nephropathy in type 2 diabetic patients [59].

- In the IDNT, 1715 hypertensive patients with nephropathy (mean serum creatinine 1.7 mg/dL [150 micromol/L]) due to type 2 diabetes were randomly assigned to irbesartan (300 mg/day), amlodipine (10 mg/day), or placebo. At 2.6 years, irbesartan was associated with a risk of the combined endpoint (doubling of the plasma creatinine, development of end-stage renal disease, or death from any cause) that was 23 and 20 percent lower than with amlodipine and placebo, respectively; the values were 37 and 30 percent lower for doubling of the plasma creatinine. These benefits were independent of the differences in the magnitude of blood pressure reduction among the groups, and the renal outcomes were best at systolic pressures below 134 mm Hg [60].

- In the RENAAL trial [61], 1513 patients with type 2 diabetes and nephropathy (mean serum creatinine 1.9 mg/dL [168 micromol/L]) were randomly assigned to losartan (50 titrating up to 100 mg once daily) or placebo, both in addition to conventional antihypertensive therapy (but not ACE inhibitors). Compared to placebo, losartan reduced the incidence of a doubling of the plasma creatinine by 25 percent and end-stage renal disease by 28 percent; the mean follow-up was 3.4 years. These benefits were again not associated with differences in blood pressure levels between the groups. Subsequent analysis of the RENAAL trial found that the most significant risk factor for progressive kidney disease was the degree of proteinuria, both initially and after six months of therapy. Additional post-hoc evaluations of RENAAL revealed the following:

- Every 10 mmHg increase in the baseline systolic blood pressure was associated with an

enhanced risk of end-stage renal disease or death by 6.7 percent.

- Lowering albuminuria within the first six months correlated with a decreased subsequent cardiovascular risk. There was an 18 percent decrease in risk of a cardiovascular event for every 50 percent decrease in the rate of albumin excretion.

- Within all categories of attained blood pressure, a larger reduction in albuminuria correlated with a progressively lower risk of end-stage renal disease.

- The presence of baseline retinopathy was associated with a poor renal outcome (increased proteinuria, decreased GFR, and development of end-stage renal disease) and a higher risk of death.

- However, all these ARB trials were underpowered and of too short duration to detect a possible cardiovascular effect.

- Olmesartan Reducing Incidence of Endstage Renal Disease in Diabetic Nephropathy Trial (ORIENT) - 577 type 2 diabetic patients with macroalbuminuria were randomized to the ARB olmesartan or placebo on top of the usual treatment (77% were treated with ACE inhibition). The study found that there was no significant effect on the primary end point—development of ESRD, death, or doubling of the serum creatinine level [62,63].

18. Combination Treatment with ACE Inhibitor and ARB (Dual Therapy)

Cost-effectiveness of early irbesartan treatment versus placebo, in addition to standard conventional blood pressure–lowering treatment, has also been demonstrated. The beneficial effect of RAAS blockade in microalbuminuric patients was also shown in the INNOVATION study in an Asian population [64]. Current evidence suggests that dual therapy is not recommended in diabetic nephropathy. The Veterans Affairs Nephropathy in Diabetes study (VA NEPHRON-D), a randomized placebo-controlled double-blind trial performed in 1448 mostly male patients with diabetic nephropathy (mean estimated GFR [eGFR], 54 mL/min/1.73 m2; mean albumin-to-creatinine ratio, 852 mg/g) showed thatcombination therapy and monotherapy groups had a similar rate of primary events (18.2 versus 21 percent). However, acute kidney injury requiring hospitalization or occurring during hospitalization was significantly more common with dual therapy (18 versus 11 percent), as was severe hyperkalaemia (9.9 versus 4.4 percent) [65]. Additional data from the diabetes subgroup of the ONTARGET trial also suggests similar findings.

19. Aldosterone Antagonists

Spironolactone [65] has been shown to decrease albuminuria and blood pressure in patients with type 1 diabetes. **Eplerenone** [67] may reduce albuminuria in patients with type

2 diabetes. **Finerenone** [68] was evaluated in a phase 2 dose-finding trial of 823 patients with type 2 diabetes treated with an ACE inhibitor or ARB. The effect of seven different doses of finerenone (ranging from 1.25 mg/day to 20 mg/day) was compared with placebo on the change in albuminuria at 90 days. A dose-dependent effect was observed, with albuminuria reductions ranging from 21 to 38 percent with doses ranging from 7.5 mg/day to 20 mg/day. The incidence of hyperkalemia with finerenone treatment was low (1.5 percent); acute reductions in eGFR were mild and were reversible after cessation of the study drug.

19.1. Aliskiren

The use of aliskiren a direct renin inhibitor, in combination with either an ACE inhibitor or ARB does not appear to preserve renal function and increases the risk of adverse events.

This was shown in the multinational Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Endpoints (ALTITUDE trial), which randomly assigned 8561 diabetic patients with either pre-existing renal or cardiovascular disease to 300 mg/day aliskiren or placebo. At baseline, the majority of patients had nephropathy; all patients received an ACE inhibitor or ARB at baseline. After a median follow-up of 32.9 months, adverse events requiring cessation of randomized therapy (usually hyperkalemia) were significantly more frequent with aliskiren (13.2 versus 10.2 percent). Due to the lack of apparent benefit and higher risk of side effects, the trial was stopped early [69].

19.2. Lipid lowering therapy

Study of Heart and Renal Protection (SHARP) investigated the effect of LDL lowering using a combination of simvastatin 20 mg and ezetemibe in 9270 patients with advanced CKD (3023 on dialysis and 6247 not on dialysis). About 20% had diabetes. The treatment reduced the incidence of major atherosclerotic events in a wide range of patients, including diabetic patients, with advanced CKD. There was no effect on mortality [70].

NKF KDOQI recommendations regarding lipid-lowering medications:

- LDL cholesterol-lowering medication (such as statin or statin/ezetimibe combination) recommended for patients with diabetes and chronic kidney disease, including those who have received a kidney transplant.

- Do not start statin therapy in patients with diabetes who are treated by dialysis.

- Atorvastatin might reduce mortality and cardiac events in patients with diabetes on haemodialysis if baseline LDL cholesterol ≥ 145 mg/dL (3.76 mmol/L). This isbased on post hoc subgroup analysis from 4D Study[71]where 1,255 patients with diabetes on haemodialysis were analysed for baseline LDL cholesterol levels. 49% died at median follow-up of 4 years

Atorvastatin was associated with reduced risk in patients with baseline LDL cholesterol ≥ 145 mg/dL (3.76 mmol/L)

Pitavastatin may reduce proteinuria compared to pravastatin in patients with type 2 diabetes and macroalbuminuria. Dose of atorvastatin does not appear to affect progression of nephropathy in patients with type 2 diabetes and early kidney disease (based on randomized trial without clinical outcomes). 119 patients (mean age 64 years) with type 2 diabetes and microalbuminuria or proteinuria were randomized to atorvastatin 80 mg/day vs 10 mg/day and followed for mean 2 years. There was no significant difference in glomerular filtration rate, creatinine clearance, serum creatinine, cystatin C, urine protein or albumin excretion between high- or low-dose atorvastatin. There was no significant difference in death or adverse events either (PANDA trial) [72].

20. Conclusion

Diabetic Nephropathy (DN) is a significant risk for not only developing end stage renal disease but also cardiovascular disease. Natural progression and history of DN is different for type 1 and type 2 diabetes. There are various risk factors for development and progression of DN, some genetic but mostly environmental. They key to halting progression of DN is likely to rest on the 3 key factors of managing **ABC** – strict glycemic control (Hb**A**1C), stringent Blood Pressure (**B**P) targets and achieving adequate **C**holesterol levels.

Specialist clinics and regular monitoring is warranted in managing these patients with DN. There is some good trial evidence with a number of different reno-protective agents for various situations and for specific population groups that may help in DN. However, we still lack specific markers that will detect DN earlier and in a more consistent basis. Also, we still don't have agents to treat DN that will make a significant impact on the long term consequences and improve prognosis for patients with DN.

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Chapter 5

Antidiabetic Effects of Antihypertensive Drugs

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Abbreviations: ACEIs: Angiotensin converting enzyme inhibitors; AngI: Angiotensin I; AngII: Angiotensin II; ARBs: Angiotensin receptor blockers; AT1R: Angiotensin II type 1 receptor; AT2R: Angiotensin II type 2 receptor; DN: Diabetic nephropathy; eNOS: Endothelial nitric oxide synthase; GLUT2: Glucose transporter 2; GLUT4: Glucose transporter 4; GSH: Glutathione; MDA: Malondialdehyde; NAD(P)H: Nicotinamide adenine dinucleotide phosphate; NO: Nitric oxide; NS: Noradrenergic system; PAI-1: Plasminogen activator inhibitor-1; (P)RR: (Pro) renin receptor; RAAS: Renin angiotensin aldosterone system; ROS: Reactive oxygen species; RR: Renin receptor; SOD: Superoxide dismutase; STZ: Streptozotocin; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α.

1. Introduction

Hypertension and type 2 diabetes frequently occur together, and because both of these conditions predispose patients to cardiovascular and renal diseases, the diabetic hypertensive patient is at an especially elevated risk of developing adverse clinical events. This chapter discusses the most useful antihypertensive drugs with antidiabetic effects for managing these challenging patients.

2. Renin Angiotensin Aldosterone System (RAAS)

Classically, RAAS is known for its role in body fluid and cardiovascular homeostasis. RAAS consists primarily of an enzymatic cascade through which angiotensinogen (Ang) is converted to angiotensin I (Ang I), which is then converted to angiotensin II (Ang II), through the action of renin and the angiotensin converting enzyme (ACE) respectively [1]. Ang II mediates its specific functions via type 1 and type 2 receptors, i.e., Angiotensin type 1 receptor (AT_1R) and Angiotensin type 2 receptor (AT_2R). Most of these functions are mediated by AT_1R, including the potent vasoconstriction, proinflammatory, pro-oxidative, proliferative and

hypertrophic effects. Moreover, advances in cell and molecular biology have allowed the recognition of other active elements of the RAAS metabolism [1].

Over the past few years, RAAS components have been found in almost every tissue, including the heart, blood vessels, kidney, brain, pancreas, adipose tissue and skeletal muscles [2]. Furthermore, a large body of evidence indicates that RAAS activation is closely correlated to both insulin resistance and beta cell dysfunction [3].

The mechanism behind this deleterious effect appears to be related to the negative regulation, exerted by Ang II through AT_1R, of several steps of the insulin signaling cascade. In addition, hyperglycemia increases the expression of RAAS components in pancreatic islets, which leads to insulin secretion modulation in beta cells, decreased adiponectin level, impaired insulin sensitivity in target tissues, inhibited glucose transporter 4 translocation and increased levels of reactive oxygen species, inflammation, and ectopic fat storage [4].

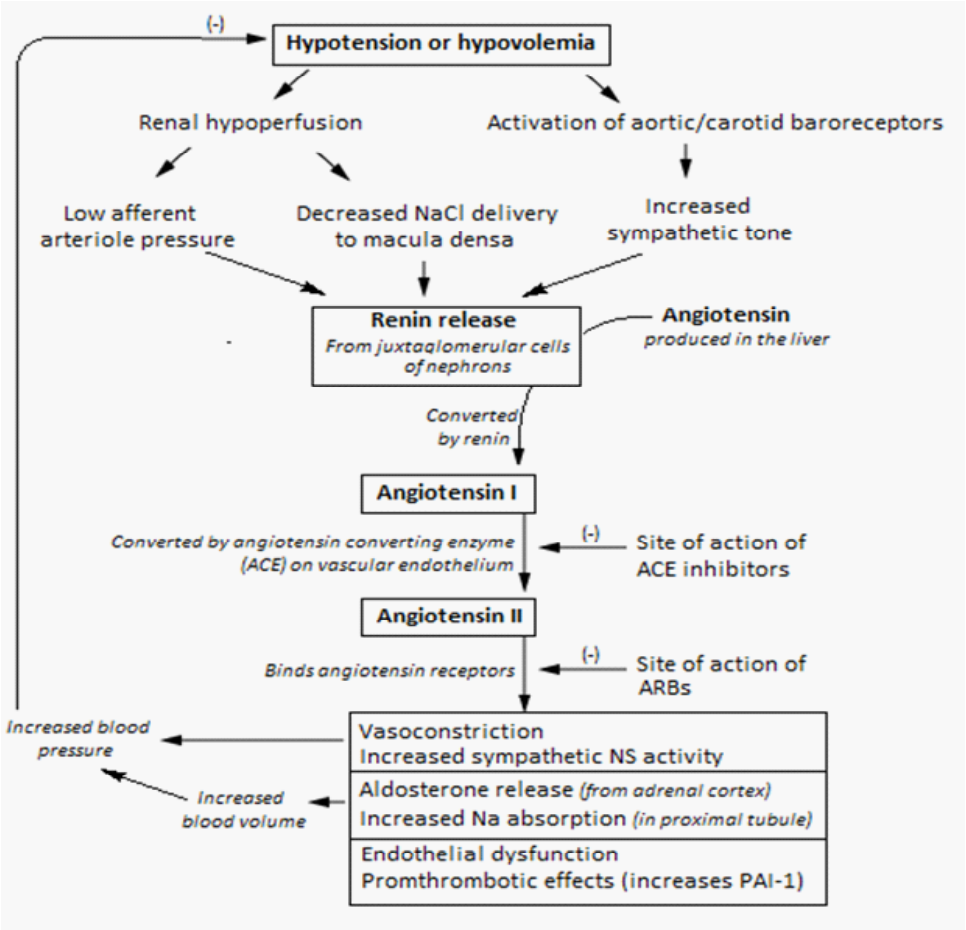


Figure 1: Flowchart showing the clinical effects of RAAS and the sites of action of ACEIs and ARBs [5].

The increase in the ACE2/Ang/Mas receptor axis could be associated with diminished insulin resistance by inducing the activation of insulin signaling pathways and counteracting the inhibitory effects of ACE/Ang II/AT1R. ACE2 gene therapy improves glycemic control in diabetic mice through a mechanism mediated by the Ang/Mas receptor because of its proven ability to potentiate the action of bradykinin [2,6].

There is an evidence that bradykinin itself may have an effect on enhancing insulin ac-

tion and signaling. Moreover, it is remarkable to note that, together with results from the beta cell injury studies, bradykinin stands out the key role in the pancreatic-duodenal homeobox 1 in prenatal development of the pancreas, as well as in the postnatal maintenance of the insulin production, and the glucose transporter 2(GLUT2) expression [7].

3. Antidiabetic Mechanisms of Angiotensin-Converting Enzyme Inhibitors and Angiotens in II Receptor Antagonists:

Growing concern about the increasing prevalence of the metabolic syndrome and type 2diabetes has generated substantial interest in the metabolic effects of antihypertensive drugs. Historically, most of the focus has been on disturbances in carbohydrate and lipid metabolism associated with diuretics and beta- adrenergic receptors antagonists [8,9,10].

However, the results of several large-scale clinical trials have recently begun to shift attention to the possibility that some of the newer antihypertensive agents may not only cause fewer metabolic side-effects than diuretics and beta- adrenergic receptors antagonists, but may also decrease the overall risk for type 2 diabetes. Given the morbidity and mortality associated with type 2 diabetes and hypertension, the availability of drugs that have antidiabetic as well as antihypertensive properties could be of considerable clinical value [11,12,13].

Antidiabetic effects of interrupting the RAASin-vitro and in-vivo experiments as well as in humans have suggested a possible relationship between the RAAS and the pathogenesis of insulin resistance. For example, studies have suggested that AngII may promote impaired glucose metabolism through its effects on insulin signaling pathways, tissue blood flow, oxidative stress, sympathetic activity and adipogenesis [14,15,16].

Thus, pharmacologic interruption of RAAS with ACE inhibitors (ACEIs) or Ang II receptor blockers (ARBs) might improve glucose metabolism by interfering with AngII generation or AngII receptor activation. These observations have begun to motivate clinical trials designed to investigate whether drugs that interrupt the RAAS can ward off the development of type 2 diabetes. Indeed, given some of the evidence accumulated to date, it is possible that pharmacologic interruption of the RAAS may someday prove to be capable of improving insulin sensitivity and decreasing the risk for diabetes. Studies on animal models and in small- and large-scale clinical trials have suggested that ACEIs may have the capacity to increase insulin sensitivity and/or to decrease the risk of type 2 diabetes [14,16,17].

Although the data are not conclusive, the results of these studies have been sufficiently interesting to motivate trials to investigate the ability of ACEIs to decrease the incidence of new-onset type 2 diabetes as a primary end-point [10,18]. Studies have suggested that the antidiabetic properties of ACEIs may be largely mediated through increases in bradykinin levels, nitric oxide and the GLUT4 glucose transporter [14,19,20].

For example, metabolic studies in animals lacking bradykinin B2 receptors and in animals treated with both an ACEI and a bradykinin antagonist strongly suggest that the insulin-sensitizing effects of ACEIs involve more than just reductions in Ang II levels [14,20]. Increases in bradykinin levels stemming from converting enzyme inhibition may improve glucose metabolism by affecting insulin signaling pathways, nitric oxide production and translocation of GLUT4 [19,21].

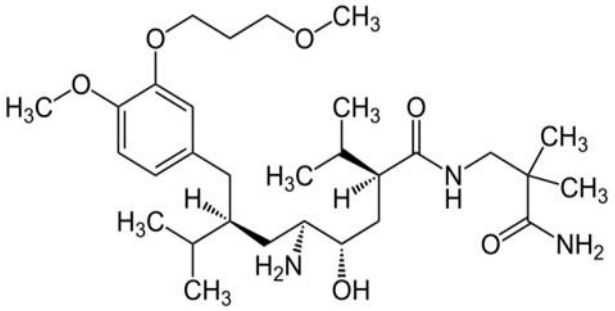
To the extent that the antidiabetic effects of ACEIs are secondary to interference with Ang II-dependent mechanisms that promote insulin resistance, one might expect ARBs to be similarly as effective as ACEIs, if not more effective; in improving insulin resistance and preventing type 2 diabetes. Paolisso et al., (1997) [22] reported that losartan-induced increases in whole-body glucose disposal were correlated with losartan-induced increases in femoral artery blood flow. However, few head-to-head comparisons have been made of the insulin-sensitizing effects of ACEIs versus ARBs and, to date, no large-scale clinical trials have compared the ability of ACEIs and ARBs to decrease the risk for diabetes [23,24,25].

Some investigators have also suggested that the inhibitory effects of AngII on insulin-signaling pathways may not be mediated by either type 1 or type 2 Ang II receptors and that another type of Ang receptor may be involved [26].

Clinical trials using ARBs have provided some indirect support for the possibility that Ang II receptor blockade per se may improve insulin sensitivity and decrease the incidence of type 2 diabetes [27].

Interventions that inhibit the activity of the RAAS like ACEIs or ARBs are renoprotective and slow progression of chronic nephropathies in animals and patients. But they have little effect on basal glucose and insulin levels, in animals without diabetes [28].

4. Aliskiren



(2S,4S,5S,7S)-5-amino-N-(2-carbamoyl-2,2-dimethylethyl)-4-hydroxy-7-{[4-methoxy-3-(3-methoxy-propoxy)phenyl]methyl}-8-methyl-2-(propan-2-yl) nonanamide

Figure 2: Chemical structure of aliskiren

Aliskiren (trade names Tekturna, US; Rasilez, UK and else where) is the first in a class of drugs called direct renin inhibitors. Its current licensed indication is essential (primary) hypertension [29].

4.1. Mechanism of action

Renin, the first enzyme in RAAS, plays a role in blood pressure control. It cleaves Ang to Ang I, which is in turn converted by ACE to AngII. AngII has both direct and indirect effects on blood pressure. It directly causes arterial smooth muscle to contract, leading to vasoconstriction and increased blood pressure. Ang II also stimulates the production of aldosterone from the adrenal cortex, which increases water and sodium reabsorption, thereby increasing plasma volume, and blood pressure. Aliskiren binds to renin and prevents the conversion of Ang to Ang I [30].

4.2. Superiority of aliskiren over ACEIs

The administration of ACEIs results in a fall in plasma AngII levels, the efficacy of ACEIs is probably limited by their inability to completely block ACE and the generation of AngII through other enzymatic pathways. However, ACEIs have other effects including interference with the breakdown of bradykinin. Long term ACEIs use is associated with a return in circulating Ang II levels following a rise in plasma renin and AngI due to the interruption of AngII feedback on renin release [31].

On the other hand, aliskiren does not affect bradykinin production and should theoretically block the actions of AngII at the AT-1R level [31].

Clinical studies indicated that aliskiren may be as effective as ACEIs and have fewer side effects. ACEIs can induce cough in susceptible individuals as a result of the increase bradykinin level. Aliskiren serves as a very good substitute for such patients [32].

Aliskiren’s ideal pharmacokineticsparameters, should be considered as added advantages [33].

In a recent study (Mahfoz et al., 2016) [34] treatment with aliskiren for one month after induction of diabetic nephropathy (DN) by Sterptozocin (STZ) resulted in euglycemia and normalized serum insulin concentration as compared to diabetic control rats. This effect was supported by the in-vitro study in which aliskiren resulted in dose dependent stimulation of insulin secretion from isolated rat pancreatic islets. In addition, aliskiren synergized gliclazide-induced insulin secretion in this in-vitro study. Furthermore, aliskiren normalized serum adiponectin concentration as compared to diabetic control rats, which was associated with decreased insulin resistance.

Authors assigned the observed antidiabetic effect of aliskiren to its ability to stimulate insulin secretion or decreased insulin resistance by normalizing serum adiponectin level, anti-oxidant or anti-inflammatory mechanisms.

Gandhi et al. (2013) [35] found that diabetic rats experienced approximately 81 % decrease in serum insulin content. However, aliskiren treatment significantly reduced blood glucose in diabetic rats. Authors explained improved insulin sensitivity effect of aliskiren by higher liver and muscle glucotransporter expression levels. In addition, Habibi et al. (2008) [36] demonstrated that renin inhibition by aliskiren attenuated insulin resistance in transgenic Ren2 rats that overexpress renin. Sun et al. (2011) [37] found an improvement in insulin resistance and lipid profile, as well as a direct antifibrotic effect in target organs in db/db mice after aliskiren treatment. Thus, a possible link between direct renin inhibition by aliskiren and insulin was suggested.

Antidiabetic effects of aliskiren treatment resulted in a renoprotective effect which was manifested by drug's ability to normalize blood urea nitrogen (BUN) and serum creatinineconcentration.

Stanton et al. (2003) and Schernthaner et al.(2008) [39,40] reported that inhibition of the rate-limiting step in the RAAS (conversion of Ang to Ang I via renin) by aliskiren which leads to potent renoprotective effect is not only caused by blocking the generation of Ang II, but also by inhibiting the effects produced via activation of (Pro) renin Receptor (P) RR. Also, aliskiren can decrease the gene expression of (P) RR, and can alter the 3-dimensional configuration of renin [40].

Although renin has been considered as the enzyme responsible for the formation of AngI and has been thought to have no direct biological actions, recent studies demonstrated that it plays a pivotal role in the development of DN by binding to (P) RR in glomerular mesangial cells [40,41]. In addition to this aliskiren was reported to improve glomerular filtration in previous studies [42,43].

Mahfoz et al., (2016) [34] reported that treatment with aliskiren resulted in antioxidant effects which was manifested by significant decrease in renal malondialdehyde (MDA) level, serum Nitric oxide (NO) concentration, increase in glutathione (GSH) level and superoxide dismutase activity (SOD). This suggests that aliskiren's renoprotective effect could be explained via com¬bating oxidative stress generated by STZ. The antioxidant effect of aliskiren is similar to the findings of earlier studies [44,45,46]. Authors suggested that increased GSH level during aliskiren treatment might be due to up-regulation of enzymatic/non enzymatic antioxidants effects or decreased reactive oxygen species (ROS) production. Aliskiren was found to decrease the NO level, which could be explained through an increase in the renal expression of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase and endothelial nitric

oxide synthase (eNOS), there by decreasing systemic and renal oxidative stress as proposed by Sonta et al., (2005) [47].

Beside the antioxidant potential, aliskiren reduced some inflammatory biomarkers level indicating its anti-inflammatory activity and this is consistant with its renoprotecting action. This was manifested by significant decrease in kidney tumor necrosis factor – α (TNF-α) and transfroming growth factor – β (TGF-β) level in diabetic rats [34].

5. Conclusion

1. It could be concluded that some antihypertensive drugs which affect the RAAS have antidiabetic effects.

2. These drugs can synergize the effect of antidiabetic drugs. Thus the dose of antidiabetic drug may be reduced in combination therapy which may reduce the occurrence of side effects. However, further clinical studies are recommended to investigate the potential of combined therapy in diabetes mellitus.

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Chapter 6

Neutrophil - Lymphocyte Ratio, as a Novel Systematic Biomarker: Predicting Intrac-eRebral Hemorrhage in Type 2 Diabetes Mellitus

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Abstract

Introduction: Chronic systematic inflammation has been suggested to be associated with the occurrence and development of cardiovascular events. Low-grade systematic inflammation persists in Type 2 Diabetes Mellitus(T2DM) patients. In addition, the risk of cerebral hemorrhage in these patients is increased compared with non-diabetic patients. Neutrophil-to-Lymphocyte Ratio (NLR) is the ratio derived by dividing the neutrophil count with the lymphocyte count from a peripheral blood sample. This study aimed to explore the relation between NLR and cerebral hemorrhage and to prove that the NLR is an independent risk factor of cerebral hemorrhage in T2DM patients.

Method: In total, 429 cases of T2DM patients were included. The patients were divided into two groups depending on the presence of cerebral hemorrhage: the cerebral hemorrhage group (n=87) and the control group (n=342). Based upon clinical and laboratory data of diabetes diagnosis, this article investigates the relationship between the NLR and risk of cerebral hemorrhage.

Results: The increase in the NLR was positively correlated with the incidence of cerebral hemorrhage in T2DM patients and might serve as an independent risk factor of cerebral hemorrhage in T2DM patients (OR 4.451 95% CI 2.582-7.672). NLR>2.58 might be useful in predicting the threshold value of cerebral hemorrhage risk in newly diagnosed T2DM patients (area under the curve 0.72, 95% CI 0.659-0.780, P<0.001)

Conclusion: As an indicator of the degree of systematic inflammation, NLR is an independent risk factor of cerebral hemorrhage in T2DM patients.

Key words: Intracerebral Hemorrhage; Type 2 Diabetes Mellitus; Neutrophil-to-lymphocyte ratio; inflammation

Abbreviation: T2DM: type 2 diabetes mellitus; ICH: Intracerebral hemorrhage; NLR: neutrophil to lymphocyte ratio; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; HbA1c: hemoglobin A1c; FPG: fasting plasma glucose; FIN: fasting plasma insulin; IL-6: interleukin-6; MCP-1: monocyte chemotactic protein-1; CRP: C reactive protein.

1. Introduction

As a common metabolic disease, type 2 diabetes mellitus (T2DM) is complicated by many cardiovascular diseases and cerebrovascular diseases, such as peripheral vascular diseases, heart failure, and intracerebral hemorrhage [1,2]. Compared with non-diabetic patients, diabetic patients are at a higher risk of hemorrhagic stroke [2]. Intracerebral hemorrhage (ICH) can cause considerable damage to the central nervous system with a high rate of disability and mortality [3]. Thus, the risk factors and pathogenesis of ICH in T2DM patients attract increasing attention.

The mechanism of ICH in T2DM remains unclear. However, studies have demonstrated that the risk of ICH is increased with chronic inflammation [4] and that diabetic patients exhibit long-term chronic inflammation [5,6]. It is easy and effective to obtain the neutrophil to lymphocyte ratio (NLR), an index of systemic inflammation [7]. Its prognostic value in tumors [8] or cardiovascular diseases [9] has been suggested by recent studies. Its association with diabetic complications has gradually received attention [10-13]. Elevated NLR is associated with risk factors of ICH, including atherosclerosis [14] and hypertension [15]. Therefore, elevated NLR itself may be used as an alternative marker to predict the pathogenesis of ICH.

The present study was designed to explore the correlation between NLR and ICH in T2DM patients and the independent risk factors of ICH in T2DM patients. This information will assist patients in taking early precautions to reduce the impact of ICH on both the health and wealth of T2DM patients.

2. Methods

2.1. Subjects

All procedures were in accordance with the ethical standards of the Helsinki Declaration of 1975 as revised in 2008. The local ethics committees approved the study protocol. In this study, 1259 diabetes patients admitted to Zhujiang Hospital and Chinese PLA General Hospital between January 2008 and December 2014 for their primary diseases were retrospectively evaluated using the electronic medical record system. Of these patients, 848 were newly diagnosed with Type 2 Diabetes Mellitus and were included for further exclusion. Type 2 Diabetes Mellitus was diagnosed based on the American Diabetes Association consulting criteria (i.e.,

fasting plasma glucose [FPG] of ≥7.0 mmol/L [126 mg/dL] and/or a 2-h post-glucose value of ≥11.1 mmol/L [200 mg/dL]).

Of these 848 patients, patients who matched the following exclusion criteria were excluded: cardiovascular diseases, myocardial infarction, heart failure, active infection, active massive hemorrhage, acute poisoning, cancer or blood diseases that affect neutrophils or lymphocytes (e.g., myeloproliferative diseases and leukemia), ICH before admission or caused by other confirmed reasons after discharge (trauma, drugs, congenital abnormalities, coagulation disorders, vasculitis, brain tumor, vascular amyloidosis, or hemorrhage secondary to ischemic stroke), or taking medication that affects neutrophils and lymphocytes (chemotherapy or radiotherapy to malignancy, granulocyte colony stimulating factor therapy or corticosteroid therapy).

After the second exclusion, 429 patients were included. Phone follow-up was implemented by using the patient database of Zhujiang Hospital and Chinese PLA General Hospital. Regarding the 75 patients who were diagnosed with ICH at Zhujiang Hospital and Chinese PLA General Hospital and 12 patients who were diagnosed with ICH at other hospitals, the patients or their family members were required to return to the hospital for a follow-up visit and information supplement. All 87 ICH patients were categorized into the ICH group. In total, 342 patients whose age and gender matched the criteria were categorized into the control group.

We followed the ADA guidelines [16] to treat T2DM and AHA/ASA guidelines [17] to treat intracerebral hemorrhage. For other complications, patients were provided standard symptomatic treatment.

2.2. Data collection

After a minimum 8-hour fast, the systolic and diastolic pressure of all patients were measured by standardized mercury sphygmomanometer (XJ11D, Shanghai Medical Instruments Co., China). Height and weight were also measured to calculate the body mass index (BMI) as weight divided by height squared (kg/m^2). A venous blood sample was obtained from the ulnar vein of each patient after clinical measurements of blood pressure, height and weight. Laboratory tests, including HbA1c, fasting glucose, fasting insulin, creatinine, uric acid, triglyceride, total cholesterol, HDL and LDL, were conducted.

The daily urine of each patient was collected to measure urinary micro albumin using a turbidimetric immunoassay (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Fasting glucose was measured using the glucose oxidase method. Fasting insulin was measured by the chemiluminescence method. An automated biochemical analyzer Synchron CX5 (*Beckman* Instruments Inc., Brea, USA) was used to measure triglyceride, total cholesterol, HDL and

LDL. HbA1c was measured using an automated high performance lipid chromatography Tosoh G7 (Tosoh Europe N.V, Tessenderlo, Belgium). Insulin resistance (IR) was assessed with a homeostasis model. HOMA-IR is FPG (mmol/L) ×FIN (mU/L)/22.5 [18].

2.3. Definitions

Intracerebral hemorrhage (ICH) was defined as a stroke for which CT scanning can identify an area of high density within the brain parenchyma with or without extension into the ventricles or subarachnoid space or an area of attenuation with ring enhancement after injection of contrast for scans performed after 1 week. MRI typically reveals an area of hypointensity or isointensity on T1-weighted images or an area of marked hypointensity on T2-weighted images. Alternatively, the origin of the hemorrhage can be demonstrated by investigation at autopsy of the cerebral parenchyma [19]. Type 2 diabetes mellitus was defined as fasting serum glucose of ≥ 7.0 mmol/L and/or non-fasting serum glucose of ≥ 11.1 mmol/L [20]. The neutrophil-to-lymphocyte ratio (NLR) was defined as a ratio of the neutrophil and lymphocyte counts. Current smoking was defined as a patient who had smoked 100 cigarettes before and smoked every day or every few days prior to admission and diagnosis of diabetes [21]. Hypertension was defined as an average SBP ≥ 140 mmHg or DBP ≥ 90 mmHg and/or current use of antihypertensive medication prescribed by a physician [22].

2.4. Statistical analysis

Statistical analysis was performed by using SPSS 16.0 (SPSS, Chicago, Illinois, USA). Continuous variables were represented as the means ± SD or medians and interquartile range. Categorical variables were expressed as percentages. Means for continuous variables between groups were compared using student's t test, the Mann-Whitney U or Bonferroni-corrected Mann-Whitney U test, ANOVA, or the Kruskal-Wallis test, when necessary. Categorical variables were compared using the χ^2 test. Pearson's correlation analysis was conducted to determine the correlation between NLR and ICH. Logistic regression analysis was used to identify the effect of each factor on ICH. Receiver operating characteristic analysis was applied to obtain the cut-off level of elevated NLR to predict ICH. A P-value of < 0.05 was considered statistically significant, and the confidence interval was defined as 95%.

2.5. Results

A population of 429 patients was included in this retrospective study. The mean age of these patients was 63.67 ± 9.74 years, and males constituted 43% of the patients. All patients were divided into two groups depending on the presence of cerebral hemorrhage: the cerebral hemorrhage group (n=87) and the control group (n=342). All patients were also categorized into quartiles by NLR. The clinical characteristics and laboratory parameters of the study population are listed in Tables 1 and 2. Patients in both groups were matched in terms of age

(P=0.416) and gender (P=0.057). In addition, no significant differences in insulin resistance, BMI, HbA1c, FPG, FINS, micro albumin, uric acid, total cholesterol, triglyceride, HDL, LDL and current smoking were noted. Significant differences were identified in systolic blood pressure. The laboratory characteristics are also presented in **Table 1**. White blood cell count, neutrophil count, lymphocyte count, neutrophil-to-lymphocyte ratio and creatinine were significantly different between the two groups.

In the Pearson's correlation analysis, elevated NLR was significantly correlated with ICH (r=0.296, P<0.001). A multivariate logistic regression model was constructed to identify the independent predictor of ICH in diabetic patients. Elevated NLR acquired when diabetes was newly diagnosed was independently associated with ICH after discharge from the hospital (OR=3.893, 95% CI 2.324-6.521, P<0.001). The adjusted OR of NLR was more obvious than current smoking (OR=2.729, 95% CI 1.453-5.127, P=0.002) and systolic blood pressure (OR=1.018, 95% CI 1.003-1.034, P=0.022) (**Table 3**).

ROC analysis was performed to determine the cut-off of NLR, WBC, neutrophil count and lymphocyte count to predict ICH in diabetic patients, as shown in Figure 1. An NLR of >2.58 may be used to predict the ICH with a sensitivity of 0.69 and specificity of 0.66 (area under the curve is 0.72, 95% CI 0.659-0.780, P<0.001). The cut-off of the remaining parameters is presented in **Table 4**.

2.6. Discussion

Diabetic patients are at high risk of atherosclerosis and corresponding complications, including coronary artery diseases, acute coronary syndrome and stroke (both ischemic and hemorrhagic) [23,24]. The diagnosis of ICH depends on tomographic instruments, including CT or MRI, to determine whether and where hemorrhage has occurred. Alternately, a diagnosis might be made according to symptoms and physical signs [25]. Remedial work could only be conducted after the occurrence of ICH, indicating that such patients must suffer from the consequences of the ICH. Therefore, research on a new marker capable of predicting ICH and preventing adverse outcome is important.

Abnormal blood sugar in diabetic patients can lead to the change of NLR which represent the long-term low-grade inflammation. This phenomenon illustrate how diabetes contributes to atherosclerosis and increase the risk of ICH.

Neutrophils represent the active nonspecific inflammatory mediator initiating the first line of defense, whereas lymphocytes represent the regulatory or protective component of inflammation [26]. Chronic hyperglycemia can promote the rise of granulocyte count by secretion of a large number of inflammatory factors and inflammatory cytokines and decrease the lymphocyte count. Meiqin Lou et al [27]'s study had shown that the NLR values of the

diabetic patients were significantly higher than those of the healsthy control. Khodabandehlou T et al [28] found that lymphocyte levels were reduced as a result of hyperglycemia in patients with diabetes and healthy subjects. And patients with diabetes mellitus have been suggested to have insufficient proliferation of lymphocytes [29] in one study by Chang FY and Shaio MF. The reduce the number of CD8+ T cells, inhibiting the anti-inflammatory environment [30], makes the persistent low-grade inflammation and a vicious cycle.

Inflammation plays an important role in the pathogenesis of atherosclerosis [31,32]. Numerous types of inflammatory factors, such as interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1), exist in the development of atherosclerotic plaques in the endothelium of vessels [33,34]. Previous studies have noted that during the development of atherosclerosis, blood neutrophil count increases, and neutrophils are attracted into the plaque to excrete pro-inflammatory products, such as elastase, myeloperoxidase, reactive oxygen species, leading to continual damage to the vessel walls [35] and impairment of endothelial function. In addition, the advanced glycation end products (AGEs) are characteristic of pro-inflammatory and potentially atherogenic functions [36,37]. Thus, the predictive value of NLR in atherosclerosis has been suggested by many studies [14, 38,39].

Through atherosclerosis and damaging endothelium of vessels, the NLR was directly and significantly associated with the risk of ICH in T2DM patients and the index also represents a reliable and dynamically stable marker of systemic inflammation that reflects the immune response and combines information of innate and adaptive pathways in a long duration.

Romero JR. et al [40] find out that carotid atherosclerosis which happened at the internal carotid artery was associated with increased cerebral microbleed (CMB)-hemorrhage-prone small vessel disease, mainly in deep regions, and the association was stronger if atherosclerosis is more severe. Common carotid artery (CCA) atherosclerotic changes may result in lower risk of hemorrhages represented by CMB due to release of prothrombotic inflammatory/endothelial cytokines.

On the other hand, recent research has proved that the NLR can independently contribute to hypertension [41], which is the most significant risk factor of hemorrhagic stroke [42]. Hypertension causes damage to the endothelium of small arteries, leading to atherosclerosis [43] and remodeling of the cerebral vasculature. Thus, hypertension causes microaneurysms at the bifurcation of arterioles. Chronic elevation of intraluminal arterial pressure can damage small vessel wall, thereby leading to eventual disruption [44]. Recent research has demonstrated that chronic low-grade inflammation plays an important role in the pathogenesis of hypertension. For example, inflammation causes endothelial dysfunction through producing NO,

which leads to a disorder between vasodilation and vasoconstriction. Thus, oxidative stress and inflammation are increased. NO production may subsequently contribute to the development of hypertension [45]. Several studies have shown that the adaptive immune response in particular contributes significantly to the pathophysiology of hypertension [46].

As a novel inflammatory marker, NLR can be acquired from routine blood counts after admission. Given its predictive value and convenience, NLR can be envisaged to be employed in screening those T2DM patients at high risk of primary ICH in the future and more potential inflammatory marker should be discussed, such as IL-6, IL-8 et al.

3. Conclusion

We conclude that as a systematic inflammation marker, NLR may be an independent risk factor of ICH. An elevated NLR can be used to predict the risk of ICH, which is helpful for the prevention of ICH. Further large-scale and long-term clinical trials are necessary to support our findings.

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5. Authors' Contributions

All authors with the same contribution to the study were responsible for the study design, data collection, and manuscript writing. Li Rui and Yue Shufan helped with the acquisition and interpretation of data and with manuscript revisions. Luo Peng analyzed the data, guaranteed this work, provided academic guidance, and took responsibility for the accuracy of the data analysis

6. Statement of Human and Animal Rights

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

7. Statement of Informed Consent

Informed consent was obtained from all patients for being included in the study.

8. Tables

Table 1: Comparison of baseline characteristics of the study population

	Control group(n=342)	ICH group(n=87)	Overall p value
Age (year)	63.86±10.31	62.92±7.06	0.320
Gender (male,%)	43%	46%	0.582
Insulin resistance	4.03±4.56	4.39±4.10	0.499
White blood cell count (10^9/L)	6.08±1.59	6.60±1.42	0.006
Neutrophil-to-lymphocyte ratio	2.30±0.58	2.74±0.55	<0.001
Neutrophil count (10^9/L)	3.77±0.99	4.11±0.93	0.004
Lymphocyte count (10^9/L)	1.71±0.51	1.53±0.35	<0.001
Platelet count (109/L)	220.9058.55	224.3556.06	0.621
Body mass index (kg/m^2)	24.34±3.65	24.66±3.57	0.462
Systolic blood pressure (mmHg)	132.35±18.54	143.20±20.84	<0.001
Diastolic blood pressure (mmHg)	78.01±9.96	82.25±11.18	0.001
Hypertension (%)	106(31%)	56(64%)	<0.001
HbA1c (%)	7.36±1.74	7.56±1.74	0.336
Fasting glucose (mmol/L)	8.52±3.95	7.97±3.82	0.247
Fasting insulin (mU/L)	11.01±9.86	12.76±11.24	0.152
Micro albumin in uria (mg/24h)	23.39±33.21	23.33±36.65	0.989
Creatinine (μmol/L)	81.13±18.81	80.90±15.41	0.916
Uric acid (μmol/L)	299.28±110.74	330.01±114.50	0.022
Total cholesterol (mmol/L)	5.24±1.24	5.48±1.54	0.127
Triglyceride (mmol/L)	2.00±1.04	2.72±1.38	<0.001
HDL (mmol/L)	1.34±0.23	1.38±0.40	0.194
LDL (mmol/L)	2.82±0.91	2.89±0.66	0.549
Current smoker (%)	48(14%)	23(26%)	0.017
Hypersensitive C-reactive protein (mg/L)	20.5±15.3	21.3±17.5	0.074
Comorbidities (%)			
Pulmonary infection	54(16%)	42(48%)	<0.001
Upper gastrointestinal hemorrhage	43(13%)	29(33%)	<0.001
Urinary tract infection	27(8%)	8(9%)	0.692
Diabetic retinopathy	10(3%)	3(3%)	0.987
Diabetic nephropathy	21(6%)	6(7%)	0.795
Drug treatment (%)			
Oral hypoglycemic agents	283(83%)	65(75%)	0.087
Antihypertensive	179(52%)	48(55%)	0.618
Antithrombotic	106(31%)	32(37%)	0.302
Stain	89(26%)	26(30%)	0.467
Duration of T2DM (Years)	9.6±4.4	8.7±6.2	0.120

Table 2: Participant characteristics by quartiles of neutrophil/lymphocyte ratio

Quartiles of neutrophil/lymphocyte ratio(range)					
	Level1	Level2	Level3	Level4	P value
	(0.21-1.94)	(1.95-2.42)	(2.44-2.88)	(2.90-8.72)	
	n=107	n=107	n=113	n=102	
Age(year)	62.76±10.65	63.52±9.93	63.48±9.31	64.98±8.98	0.416
Gender(male,%)	71(66.4%)	54(50.5%)	64(56.6%)	53(51.9%)	0.057
Prevalence of ICH	15(14%)	19(18%)	19(18%)	34%(31%)	0.034
Insulin resistance	3.73±3.93	3.79±3.02	4.61±6.54	4.27±3.29	0.418
Whitebloodcellcount(109/L)	5.56±1.46	6.09±1.40	6.46±1.75	6.65±1.42	<0.001
Neutrophilcount(109/L)	1.58±0.28	2.19±0.14	2.65±0.14	3.16±0.18	<0.001
Lymphocytecount(109/L)	3.14±0.84	3.80±0.83	4.028±0.96	4.41±0.86	<0.001
Platelet count(109/L)	223.66±58.56	222.94±56.79	221.73±57.09	217.88±60.35	0.894
Bodymassindex(kg/m2)	2.03±0.61	1.74±0.38	1.5193±0.36	1.39±0.27	0.958
Systolicbloodpressure(mmHg)	24.45±3.79	24.37±3.80	24.28±3.37	24.54±3.60	<0.001
Diastolicbloodpressure(mmHg)	128.56±15.62	134.34±18.67	135.19±19.41	140.33±22.33	0.001
HbA1c(%)	7.55±0.79	8.06±1.15	7.90±0.97	8.05±1.12	0.911
Fastingglucose(mmol/L)	7.36±1.68	7.33±2.00	7.49±1.60	7.43±1.66	0.462
Fastinginsulin(mU/L)	8.06±3.54	8.85±4.16	8.50±4.18	8.19±3.75	0.47
Microalbumininuria(mg/24h)	10.77±10.61	10.48±8.87	11.78±12.11	12.46±8.51	0.102
Creatinine(μmol/L)	76.25±17.50	81.14±18.45	81.91±16.69	85.15±19.15	0.005
Uricacid(μmol/L)	291.25±105.06	304.16±115.89	309.46±105.80	317.51±121.43	0.384
Totalcholesterol(mmol/L)	5.29±1.43	5.19±1.00	5.23±1.012	5.44±1.70	0.53
Triglyceride(mmol/L)	2.12±1.16	2.04±1.04	2.05±1.14	2.41±1.24	0.068
HDL(mmol/L)	1.34±0.22	1.34±0.24	1.33±0.23	1.36±0.38	0.83
LDL(mmol/L)	2.718±0.88	2.90±0.72	2.81±0.91	2.92±0.94	0.299
Current smoker(%)	19(17.7%)	18(16.8%)	20(17.7%)	14(13.7%)	0.847

Table 3: Independent predictor of ICH in multivariate logistic regression analysis

	Unadjusted OR(95%CI)	**P value**	**Adjusted OR(95%CI)**	**P value**
White blood cell count (109/L)	1.224(1.059-1.416)	0.006	1.059(0.891-1.259)	0.516
Neutrophil-to-lymphocyte ratio	4.135(2.567-6.662)	<0.001	3.717(2.216-6.233)	<0.001
Current smoker (%)	2.201(1.250-3.876)	<0.001	2.850(1.502-5.407)	0.001
Systolic blood pressure (mmHg)	1.027(1.015-1.039)	0.001	0.995(0.973-1.017)	0.632
Diastolic blood pressure (mmHg)	1.040(1.016-1.064)	0.006	1.011(0.981-1.041)	0.472
Hypertension (%)	3.967(2.419-6.507)	<0.001	3.618(1.565-8.362)	0.003

Table 4: Receiver operating characteristic (ROC) analysis for neutrophil-to-lymphocyte ratio to predict ICH (area under curve is 0.720)

Parameters	AUC	Cut-off value	Sensitivity	Specificity	Youden index
WBC	0.619	6.350	0.586	0.655	1.241
NLR	0.720	2.579	0.690	0.664	1.354
Neutrophil	0.608	3.750	0.667	0.515	1.182
Lymphocyte	0.400	1.797	0.202	0.620	0.827

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