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**Name of Journal:** *World Journal of Meta-Analysis*

**Manuscript NO:** 82569

**Manuscript Type:** MINIREVIEWS

### **Lipocalin-2 (NGAL) as a Biomarker of Diabetic Nephropathy**

Kiran Dahiya, Praveen Prashant, Rakesh Dhankhar, Kumud Dhankhar, Sushil Kumar, Sonia Vashist

#### **Abstract**

Diabetes is a major global public health issue. The prevalence of type 1 diabetes is comparatively static, as hereditary and genetic causes are involved, while type 2 diabetes ( T2D) prevalence is increasing day by day. T2D is associated with chronic complications, including diabetic neuropathy, nephropathy, retinopathy, and other complications like the diabetic foot. Diabetic nephropathy is the main complication of both types of diabetes. Diabetic nephropathy can be diagnosed with routine laboratory tests, microalbuminuria >300 mg/24 h, and a gradual decrease in glomerular filtration rate. As the appearance of microalbuminuria is a late manifestation, an early marker of renal damage is needed. Lipocalin-2, or neutrophil gelatinase-associated lipocalin (NGAL), is a small protein purified from neutrophil granules and a good marker for kidney disease. NGAL is a transporter protein responsible for many physiological processes, such as inflammation, generation of the immune response, and metabolic homeostasis. NGAL has been reported to depict the early changes in renal damage when microalbumin was still normal in urine. Therefore, the role of NGAL in detecting diabetic nephropathy (DN) and understanding its mechanism is evident, thereby establishing it as a potential early marker of DN.

#### **INTRODUCTION**

<sup>1</sup> Diabetic nephropathy (DN) is a late but one of the chronic complications of diabetes, and it affects more than 40% of both type 1 diabetes mellitus (T1D) and type 2 diabetes mellitus (T2D) and may lead to end-stage renal disease (ESRD) as reported worldwide. Diabetic nephropathy can be diagnosed clinically based on a gradual decrease in glomerular filtration rate (GFR) and an increase in albuminuria >300mg/24 h which is shown to be associated with cardiovascular complications. An early diagnostic and prognostic marker is still needed to detect DN early for better treatment outcomes and predictive value. [1-2].

The current diagnostic markers of DN, viz. microalbuminuria and serum creatinine levels, have questionable reliability even when specific indicators like creatinine clearance or ratio of creatinine and albumin in 24-hour urine samples are used. Microalbuminuria can be associated with other physiological and pathological conditions such as exercise, diet, infections, and dehydration [3]. Serum creatinine levels vary according to age, gender, hydration, muscle mass, and kidney conditions. Serum creatinine levels are seen raised later in the advancing disease. So the reliability of these markers in early renal damage detection is questionable [4-10].

**SELECTION OF A RENAL BIOMARKER:** The characteristics of a biomarker shall be considered to determine its usefulness. Its measurement should be easy and accurate, and results should be reproducible. It should also indicate an early renal injury, and the response to the treatment, cost-effectiveness and availability should be taken care of. It should be able to be applied to a large population and augment the disease's clinical diagnosis and prognosis [11].

The commonly used markers of acute kidney injury (AKI) and renal dysfunctions are plenty which may be extrapolated to diabetic nephropathy. The biomarkers for oxidative stress include 8-hydroxy-2'-deoxyguanine (8-OHdG) as a novel but controversial marker of deoxyribonucleic acid (DNA) damage; pentosidine, 2,4-dinitrophenylhydrazine (DNPH) and advanced oxidation protein products (AOPP) for protein injury, and F2-is prostaglandin and 4-hydroxy-nominal (HNE) for lipid injury. The glutathione-s-transferase (GST), an enzyme-like protein, and lipid peroxidation

(LPO) are markers of the glutathione antioxidant system and lipid peroxidation. Certain other biomarkers of inflammation, like cytokines and a variety of chemokines too, are essential biomarkers of AKI and kidney dysfunction and include interleukin-8 (IL-8), tumor necrosis factor-  $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1) and interferon-inducible protein-10 (IP-10). The renin-angiotensin-aldosterone system (RAAS) biomarkers are also used as kidney injury markers [12-14].

Biomarkers for damage to glomerular filtration membranes include podocin, synaptopodin, and nephrin urinary mRNA levels. Basement membrane injury markers like type IV collagen levels are substantially higher before microalbuminuria and associated urinary albumin and serum creatinine [15-16]. The biomarkers of endothelial cell injury, like vascular endothelial growth factor (VEGF), von Willebrand factor (vWF), and intercellular adhesion molecule-1 (ICAM-1), are found raised in patients with diabetic nephropathy [17-20]. The biomarkers for mesangial expansion and fibrosis are also crucial, as DN is seen with extracellular matrix alterations and mesangial expansion, *e.g.*, transforming growth factor-  $\beta$ 1 (TGF- $\beta$ 1) and pigment epithelial-derived factor (PEDF) [21-22]. The alteration of renal function is associated with glomerular and renal tubular dysfunction [23]. Transferrin, ceruloplasmin, and immunoglobulin G (IgG) are early biomarkers for glomerular dysfunction. The renal tubular dysfunction markers include  $\alpha$ -1 microglobulin, retinol-binding protein 4 (RBP4), lipocalin-2, N-acetyl-  $\beta$ -D-glucosidase (NAG), kidney injury molecule-1 (KIM-1) and heart-type fatty acid binding protein (H-FABP). [24-27].

**Lipocalin-2:** The neutrophil gelatinase-associated lipocalin (NGAL) or lipocalin-2 is a small protein purified from neutrophil granules and is considered a good marker for acute kidney injury and kidney disease. It belongs to the lipocalin family and is encoded by the lipocalin-2 (LCN2) gene on chromosome 9 [28-30]. NGAL is a transporter protein responsible for many physiological processes, such as inflammation, the generation of an immune response, and metabolic homeostasis. Several studies have reported the role of lipocalin-2 in renal diseases suggesting its role as a novel biomarker of acute renal injury and chronic kidney disorders. A few studies have also

demonstrated its inverse relation with serum creatinine in T1D and T2D, although these patients had normal albuminuria. In patients with diabetic nephropathy, <sup>1</sup> NGAL levels were significantly higher in serum and urine, which correlated with the estimated glomerular filtration rate (eGFR) inversely. However, these patients had normal albuminuria, implicating the potential role of NGAL as a diagnostic biomarker for DN.<sup>[29-33]</sup>

<sup>13</sup> **EXPRESSION OF NGAL IN BODY TISSUES:** NGAL is expressed in several body tissues, including the kidney, liver, lungs, trachea, small intestine, bone marrow, prostate, non-neoplastic breast tissue, macrophages, and fat tissues. Expression of NGAL is seen in fetal skin as early as the 20<sup>th</sup> intrauterine life in the epidermis and later concentrated around hair follicles only <sup>[32,34]</sup>.

The normal concentration of NGAL in serum averages 20 ng/mL, while in urine, it is 20 ng/mL. Its low molecular weight and positive charge make it undergo filtration, so renal clearance is seen as the primary regulator of the concentration of NGAL. <sup>[35-36]</sup>

**FUNCTIONS OF NGAL (LIPOCALIN-2):** As part of transport proteins, lipocalin-2 is also seen in many physiological conditions of the body involved in the innate immune response. It is generated through neutrophil degranulation and thus released at the site of bacterial infection for bacterial sequestration. Iron transport is another role of NGAL as it is accumulated in the cytoplasm, and iron-responsive genes are stimulated in response to this increased concentration. Apo-NGAL is responsible for transporting chelated iron from the inside to the extracellular matrix. Apo-NGAL binds to the 24p3 receptor and internalizes to bind with the cellular siderophore, thus transporting it out of the cell. It signalizes the apoptotic cascade to start due to the expression of the pro-apoptotic protein Bim. The initiation of programmed cell death, whether under normal or abnormal circumstances, depends on the Bim protein. Its activation is precisely regulated at various levels to ensure its proper functioning. Bim is essential in preventing autoimmunity during normal immune responses; however, excessive activation can lead to chronic inflammation and tumor development. In nerve cells, the overexpression of Bim can result in neurodegenerative diseases such as Alzheimer's

and Parkinson's. On the other hand, cancer cells typically inhibit Bim expression from facilitating their proliferation and metastasis. [29,37-44].

**NGAL AND RENAL DAMAGE:** The low molecular weight of NGAL makes it easily filterable through the glomerulus and later reabsorbed in proximal tubules. If renal tubular damage starts, the reabsorption alters, and thus excretion of NGAL starts early; the epithelial damage results in increased production, and thus NGAL concentration rises in the serum and urine [45].

**OVEREXPRESSION OF NGAL IN OTHER DISEASES:** Several inflammatory and metabolic disorders are seen with altered concentrations of NGAL. Inflammatory conditions like pancreatitis, meningitis, psoriasis, and myocarditis are seen with increased NGAL expression. In certain autoimmune diseases like psoriasis, NGAL mRNA levels were raised ten times or more. NGAL levels have been reported to be considerably higher in viral infective diseases but markedly lower in human immunodeficiency virus (HIV) patients who were not receiving therapy than in healthy controls [46,47]. Higher levels of NGAL were found to be associated with anemia independent of eGFR and other parameters like myeloperoxidase and high-sensitivity C-reactive protein (hs-CRP) [36].

## NGAL

Figure 1: Alteration of NGAL levels in different diseases; levels increase in all except a few cancers where levels are found to decrease.

<sup>15</sup> Abbreviations: AKI acute kidney injury; CKD chronic kidney disease; CVD cardiovascular disease; DM diabetes mellitus.

**NGAL IN CANCERS:** NGAL's possible role in various cancer models has been studied and is suggested to be both beneficial and detrimental. The <sup>16</sup> nuclear factor kappa B (NF-

11  
κB) signaling pathway regulates the transcription of NGAL, and the mitogen-activated protein kinase (MAPK) pathway may cooperate with NF-κB to upregulate the expression of NGAL. Moreover, epigenetic modifications might significantly initiate NGAL expression in the tumor cells [28,48-56]. It may explain the increased levels of NGAL in most cancers. It remains to identify the specific molecular forms of NGAL (in serums and cells) associated with a particular cancer type (solid or liquid) [48]. Functionally NGAL appears to exhibit all the significant events of tumorigenesis, including tumor proliferation, tumor cell survival, distant migration, local invasion, tumor angiogenesis, and resistance to anti-cancer drugs [57]. NGAL protein and mRNA levels are quantitatively measured in body fluids like blood, urine, and tissues and found overexpressed in various cancers like ovarian, endometrial, bladder, liver, breast, brain, lung, pancreatic, colorectal, and several other solid tumors [48-50,54]. The NGAL complex may help assess the tumor staging in endometrial cancers before surgical treatment. 6 The NGAL complex is found in blood tumor cells in patients with different types of leukemia. [55,58-60].

**METABOLIC DISORDERS AND NGAL:** In metabolic diseases, including obesity, kidney disorders, and pre-eclamptic subjects, NGAL levels were significantly higher in animal models and obese human subjects [61-63]. Diabetes mellitus type 2 is characterized by inflammatory processes in the whole body, resulting in endothelial dysfunction. 9 Pro-inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF), as well as chemokines and adhesion molecules, have been shown to contribute to vascular complications in T2DM. In diabetes mellitus type 1, an early predictive role of NGAL as a biomarker for nephropathy and incipient cardiovascular morbidity before and independent of microalbuminuria [6].

#### **NGAL IN DIABETIC NEPHROPATHY:**

5 The plasma NGAL (pNGAL) is filtered by the glomerulus and can be almost reabsorbed in the proximal tubules. The chance detection of urinary NGAL (uNGAL) and pNGAL in animal and human subjects of renal injury have led to evaluating NGAL as an early noninvasive biomarker in human acute and chronic kidney injury in numerous research

studies. Lipocalin-2<sup>3</sup> is, therefore, one of the most promising early, next-generation biomarkers in the field of AKI. The glomerular basement thickening and mesangial expansion have been reported in several studies. The pathogenesis of DN is associated with glomerular and renal tubular interstitial injury. The primary mechanism of NGAL clearance from the blood is *via* megalin-dependent endocytosis in the proximal tubules of the kidney. Therefore,<sup>10</sup> urinary excretion of NGAL is only expected when there is proximal renal tubular injury that prevents NGAL reabsorption or increased de novo NGAL synthesis. The NGAL protein secreted into the urine from the distal nephron segments is predominantly monomeric and differs from the dimeric<sup>5</sup> NGAL originating from neutrophils. The overexpression of NGAL in the distal tubules and its rapid secretion into the urinary tract aligns with its role as an antimicrobial strategy. Furthermore, recent evidence suggests that NGAL may also promote cell survival and proliferation, given the documented<sup>5</sup> apoptotic cell death in distal nephron segments in various animal and human models of AKI. [6,28,62]

Various<sup>3</sup> proteomic and transcriptomic research has identified the NGAL gene (LCN2 gene) as one of the most upregulated genes and one of the most highly induced proteins in the kidney very early in the course of acute kidney disease in animal as well as human models [63,64]. NGAL is a novel marker for the diagnosis of DN. It is a marker of kidney injury or any other condition affecting the functions of the kidneys. Early diagnosis of AKI is often challenging and complicated, as suitable early markers of renal damage and kidney function are scarce. NGAL,<sup>14</sup> being an early marker of AKI, overcomes such limitations and seems to demonstrate its role in the diagnosis at an early stage [65,66].

Various studies have reported increased urinary and serum levels of NGAL in acute kidney injury. NGAL's role as an early biomarker to diagnose DN, even earlier to incipient nephropathy, can be seen as a tubular injury marker. Both pNGAL<sup>1</sup> and uNGAL can predict early tubular damage and can be used as noninvasive tools for diagnosing, staging, and watching over progressing DN [67]. Subclinical and early kidney injury can be seen in children with T1D with normal renal function. The pNGAL

and uNGAL derangement, low-range albuminuria, and normal eGFR can indicate early kidney injury even in optimal glycaemic control. pNGAL and uNGAL in these changes result from tubular injury [68].

In non-terminal chronic kidney disease, NGAL can be used as a novel, independent renal predictor of CKD progression along with the severity of the renal disease. The urinary NGAL can be used as a marker for the early detection of diabetic nephropathy, and its mean value has been observed to correlate with the degree of renal impairment.

The parallel elevation in uNGAL with disease severity or with increasing stages of CKD supports the hypothesis of active tubular production, excluding a passive consequence of reduced renal clearance capacity. Urinary NGAL has been reported to correlate positively with urine albumin/creatinine ratio (ACR), duration of diabetes, hemoglobin A1C, and dyslipidemia. As the positive urine NGAL results were found even in normoalbuminuric patients, uNGAL can be used as an early biomarker for DN in normoalbuminuric patients, especially those with long-standing and uncontrolled diabetes [28,69-72]. Urinary NGAL levels may help monitor the status and treatment of diverse renal diseases reflecting defects in the glomerular filtration barrier, proximal tubule reabsorption, and distal nephrons [34].

It was appreciated that uNGAL is produced in response to ischemia, toxins, or inflammation in the tubular epithelial cells. For each 300 ng/mL increase in uNGAL, an increased risk for the resultant outcome of CKD (due to T1D and T2D) progression, end-stage kidney disease, or death in CKD patients is seen. Urinary NGAL of the microalbuminuric group increased way higher than the normoalbuminuric group [73-75].

The plasma levels of NGAL and IGFBP4 have been appreciated to be higher in patients with diabetic nephropathy. Regular follow-up and monitoring before the symptomatic presentation of DN can be carried out with serial monitoring of uNGAL levels, but defining the baseline concentration of NGAL in patients is required [76-78].

The uNGAL may be a more specific marker of active renal tubular epithelial damage and tubulointerstitial inflammation, whereas pNGAL may be more indicative of the renal (and possibly extra-renal) vasculature state, including glomerular filtration ability.

Increased level of NGAL as an endogenous filtration biomarker in type 2 diabetic patients is considered a predictive biomarker for early detection of diabetic nephropathy. The uNGAL was found to be higher in patients with macroalbuminuria than normoalbuminuria, especially in those with long-standing, uncontrolled diabetes and dyslipidemia [79-82]. The serum NGAL (sNGAL) showed an excellent diagnostic value comparable to uNGAL [83].

Urinary NGAL has a positive association with microalbuminuria and can be a noninvasive tool for diagnosing and monitoring the progression of diabetic nephropathy. Urinary NGAL measurement is more sensitive than microalbumin, detecting early renal involvement in patients with diabetes mellitus. The uNGAL and creatinine ratio (uNCR) might prove promising in identifying cases with a high clinical suspicion of diabetic kidney disease and in patients with confirmatory biopsy. T2D patients with increased uNCR may have worse outcomes and higher chances of DN complications. However, pNGAL rises markedly with the reduction in GFR, resulting in many false positive inclusions of acute kidney injury in chronic patients. So along with eGFR, the urinary NGAL and plasma brain natriuretic peptide (BNP) should be used in chronic kidney disease patients to assess AKI [69,84,85].

The increase in uNGAL and cystatin-C levels directly proportionated with microalbuminuria in diabetic patients. T2DM patients with early diabetic nephropathy had high uNGAL and cystatin-C levels. NGAL reflects tubular damage, and nitric oxide (NO) may be used as an angiogenic and oxidative stress marker. Using specific biomarkers along with NGAL can increase its diagnostic efficacy in differentiating renal causes from other clinical conditions [85-88]. uNGAL may be a more specific marker of active renal tubular epithelial damage and tubulointerstitial inflammation, whereas pNGAL may be more indicative of the renal (and possibly extra-renal) vasculature state, including glomerular filtration ability [89].

However, some studies have shown that exosomal-NGAL (NGAL-E) is a better marker than free-NGAL in T1DM. NGAL was present in subjects' urinary enriched extracellular

vesicle fraction (NGAL-E); however, NGAL-E did not correlate with glycated hemoglobin and albumin/creatinine ratio in the early stages [90].

NGAL was readily detected in the urine after anti-neoplastic drug administration in a dose- and duration-dependent manner. By comparison, urinary NGAL excretion following cisplatin administration was quantified within 96 h of drug administration so that it can be used as an early marker of kidney injury in cancer subjects very early, showing its efficacy as an early marker in other pathologies leading to renal dysfunction [91,92].

A metanalytical study also concluded <sup>3</sup> that NGAL is a potential diagnostic marker for patients with DN and that its diagnostic value for microalbuminuria and macroalbuminuria is superior to that for microalbuminuria [93]. Several studies collectively and strongly support using NGAL as a biomarker for predicting AKI. However, the <sup>1</sup> lack of published studies that adhere to diagnostic study guidelines, heterogeneity in AKI definition, the lack of uniformly applicable cut-off values, and variability in the performance of commercially available NGAL assays is a big challenge to establish its role concretely [94]. The specificity and sensitivity of NGAL were found to be moderate to excellent in various studies in various conditions, including indoor and outdoor patients, as a good predictor of acute kidney injury [10,63,95-98]. Although some limitations are reported, NGAL (sNGAL and uNGAL) can be prognostic of renal damage even in the case of subclinical or modest renal damage that can only be diagnosed by creatinine studies late in the course of the disease [99].

## **CONCLUSION**

The studies reported in the present review describe the role of NGAL in nephropathy, particularly diabetic nephropathy. Early detection of renal changes is vital for diagnosis and prognostic purposes. NGAL is an important renal dysfunction marker. Though its role in other conditions like infections, metabolic disorders, and cancers is already established, its function in nephropathy is also promising, as it increases significantly before other usual markers appear in the urine and blood.

### **Figure Legends**

Figure 1: Alteration of NGAL levels in different diseases; levels increase in all except a few cancers where levels are found to decrease.

Abbreviations: AKI acute kidney injury; CKD chronic kidney disease; CVD cardiovascular disease; DM diabetes mellitus.

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