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Neutrophil gelatinase associated lipocalins (NGAL) as an inflammatory biomarker: A special reference to renal system

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Abstract
Inflammation is a part of protective response of body to any harmful etiological agent. Inflammation is mainly characterized by local responses such as rubor, calor, dalor and functio- lechio. It is mediated by variety of cytokines and inflammatory warriors such as neutrophils, macrophage, and eosinophils at the site of injury. Apart from the mediators of inflammation, enhanced protein synthesis also occurs at the site of injury to combat the spread of infection. This newly synthesized protein molecule participates along with phagocytic warriors, mediates and resolutes inflammatory site. One of such inflammatory protein is NGAL, which belongs to lipocalin family, synthesized at site with various signaling cascade. Being part of inflammation, their levels also gets elevated in serum and urine, which rules out kidney injury, hence they exhibit a dual face of inflammatory mediator and biomarker of kidney disease (1). With this idea in the upcoming review we summarize the role of NGAL as a inflammatory mediator and its role in disease progression.

Keywords: NGAL, Inflammatory sequence, lipocalins, Granule exocytosis, Biomarker of renal disease

Introduction
NGAL is neutrophil gelatinase associated lipocalins also known as siderocalins or uterocalins (being secreted in pregnant uterus) which belongs to a large group of secreted glycoproteins called Lipocalins [19]. NGAL expression initially identified in mature neutrophils, after that with occurrence of inflammation all over body its expression has been ellucited in multitude of tissues [13]. Recently its expression also seen in renal endothelial cells with event of kidney injury. This lipocalin family shows great diversity at the sequence level. Lipocalins classified as kernel and outlier lipocalins with their characteristics sequence motifs. Kernel lipocalins shares three conserved motifs while the outlier lipocalins shares only one sequence. Apart from this sequence motifs, lipocalins shares highly conserved crystal structure. Together with other two families of ligand binding proteins (Fatty acid binding protein and Avidins) lipocalin form part of superfamily *Calycins*. Lipocalins can bind a small hydrophobic molecule, bind to specific cell surface receptors, forming complex with soluble macromolecules. Earlier lipocalin family was thought to be just a transport protein (retina transport) but currently its immense roles in prostaglandin synthesis, olfaction, pheromone transport, immune response, as carrier protein in exogenous and endogenous compounds has been explored. It has been proposed that they have array of physiological functions, some lipocalins also take part in pathological condition like inflammation apart from its physiological significance [28]. The ligand for NGAL is unknown but binding of bacterial formyl peptide methonyl leucyl phenyl alanine (FMLP) may be suspected [3]. This protein is also believed to bind a small lipophilic substance such as LPS and function as modulator of inflammation [7]. This article emphasis on NGAL role in mediation of inflammation its molecular mechanism and its role in transtition from acute to chronic kidney disease.

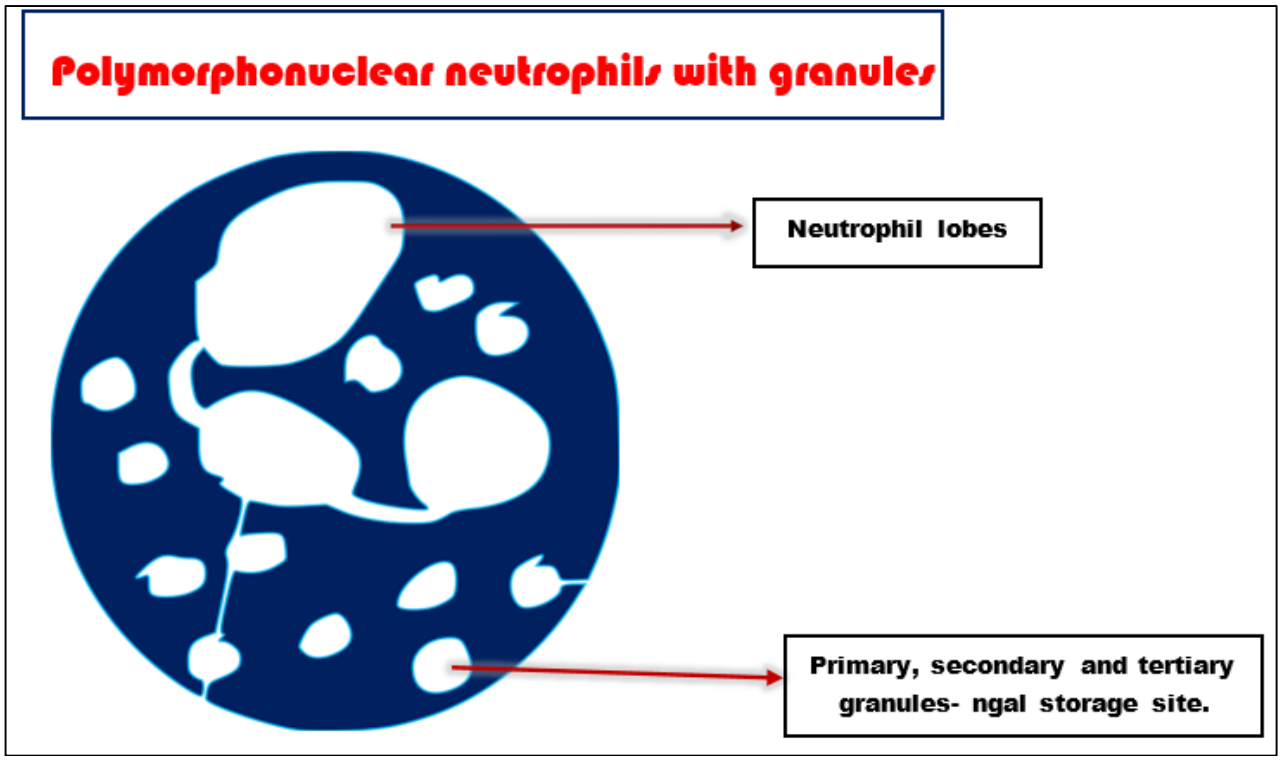

Fig 1: Polymorphonuclear neutrophils with granules

NGAL in Neutrophil

Neutrophil also known as first line of defense /scavenger/phagocytic warrior which mainly initiates and mediates inflammation. Inflammation is vascular and cell mediated changes at the site of injury. Neutrophil consists of lobed nucleus with granules in cytoplasm. Neutrophil granules are of three types namely primary, secondary, tertiary granules and secretory vesicles which are exocytosed as a part of phagocytic activity [8]. Primary granules consist of enzyme myeloperoxidase, secondary granules consist of lipocalin colocalized with lactoferrin B complex [7] and tertiary granules consists of gelatinase granules. This NGAL has been synthesized in different subcellular compartment at metamyelocyte stage of neutrophil synthesis. During resting stage this NGAL is packaged in different subcellular compartments by means of covalent attachment [23] With the onset of inflammatory process the neutrophils get activated and NGAL is released at site of inflammation as a assembled package. This emphasis that NGAL (glycoprotein) synthesis occurs along with granules of neutrophils, get stored in different subcellular compartments and exocytosed along with neutrophil degranulation process [32].

Inflammatory Sequence

Inflammatory sequence can be viewed in two aspects: one is changes that occurs at the site of injury/site of etiological approach and second is changes that ensues injury (alterations in the permeability of blood vessels and reaction of inflammatory cells). Here Kidney is a suitable model, since kidney receives most of cardiac output and highly exposed to toxicants with process of elimination from the body, renal system is more prone for Injury and inflammation often. Any noxious stimuli to renal endothelial cells, there is down regulation of renal endothelial anchoring proteins such as NETRIN 1 and Sphingosine molecules [5]. Downregulation of this proteins leads to loosening of endothelial structure there by etiological agent gives free ride into lumen of renal cells without any hindrance. As the etiological agent gains access into lumen they release a pathogen associated molecular pattern (PAMP) and Endothelial cells also expresses damage associated molecular pattern (DAMP) which aids in entry of pathogen into lumen. This PAMP and DAMP interacts with TOLL like receptors on sentinel cells like macrophage and dendritic cells this cause them to release cytokines and chemokines which attracts neutrophils to site of injury. This released mediators also enhances expression of selectins on endothelial cell surface to aid in increased neutrophil recruitment [25]. The released cytokines through bloodstream stimulates release or crowding of phagocytic cell neutrophil towards site of injury in order to evade pathogens [27].

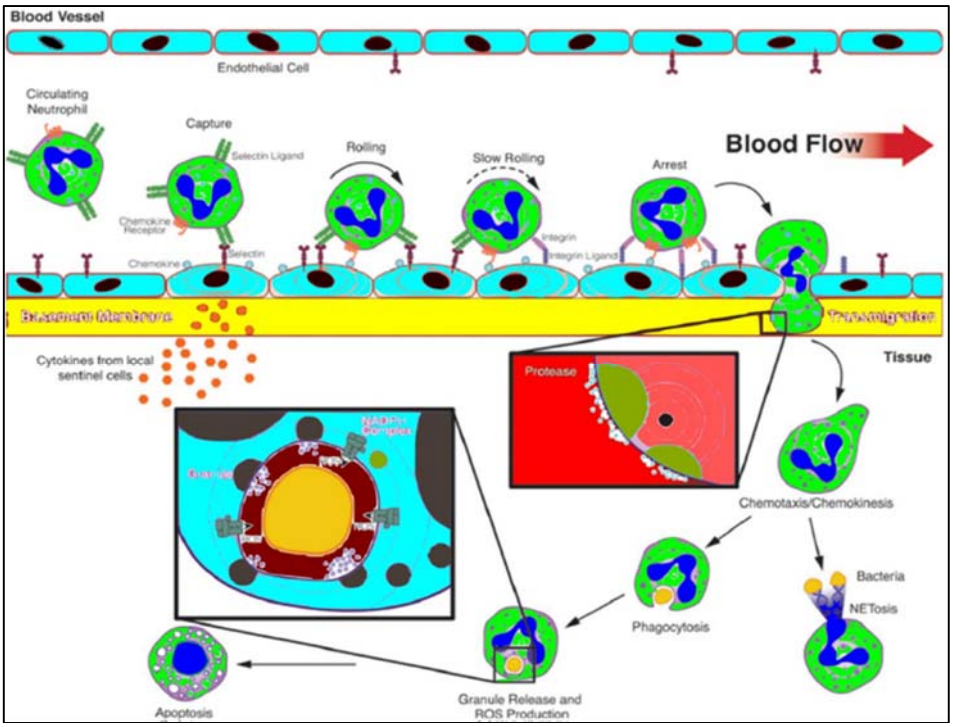

Fig 2: Conventional method of neutrophil recruitment in vascular unit: Neutrophil comes out of blood vessel by means of process such as adhesion, rolling, arrest and transmigration that occurs with all inflammatory process.

This released cytokines creates a concentration gradient which helps in navigation of neutrophil from blood vessel to reach the destination. Initially circulating leucocyte tethered loosely to endothelium through interaction of selectin on leucocytes or endothelium. This selectin mediated cell to cell interaction cause leucocyte to roll on endothelium which normally occurs. An alternative method of neutrophil recruitement occurs in specialized beds of glomerular capillaries in which P-selectin adherent to endothelial cells serves as a bridge between endothelial cell and neutrophil a process termed as secondary capture (Zarbock. A., *e*, 2011). This contact may transduce signals in neutrophil that mobilizes secretory vesicle [26]. Integration of membrane of secretory vesicle and its associated CD11b/CD18 enhances neutrophil firm adhesion to endothelium. Exocytosis of gelatinase granules helps in degrading type IV collagen in basement membrane. The rolling leucocytes becomes immobilized by interaction of leucocytes integrins and Vascular cell adhesion molecule (VCAM-1) and Intersitial cell adhesion molecule (ICAM-1) [10]. More and more of leucocytes by means of adhesion, rolling and chemotaxis exit from blood vessel reaches site of injury.

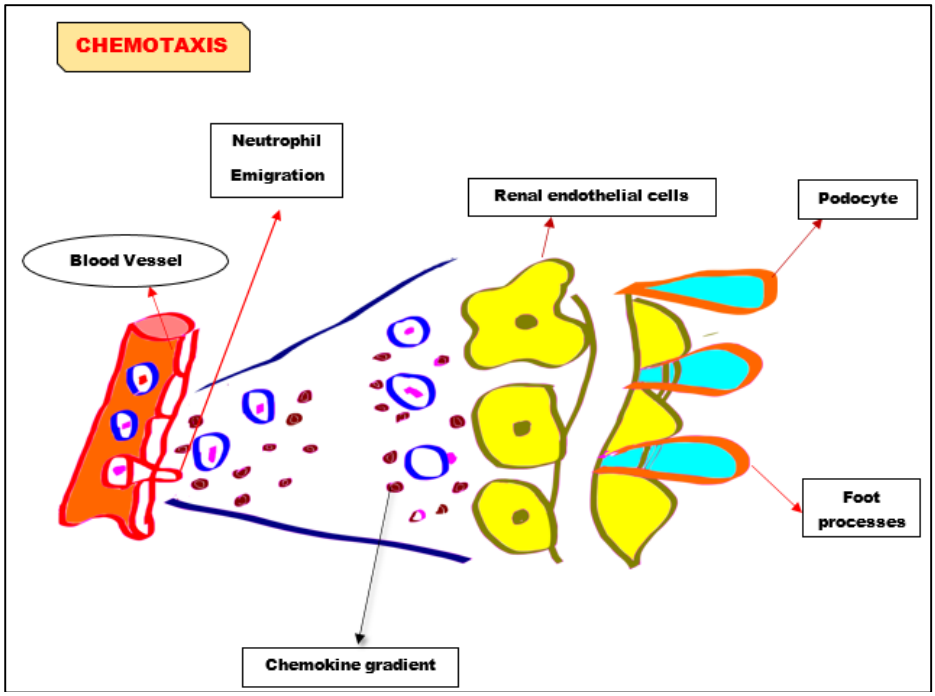

Fig 3: Neutrophil after Coming Out Of Blood vessel Moves Towards The Inflammatory Site By Sensing The Chemokine Concentration

ICAM-1 expression is higher in endothelial cells, mesangial cells, epithelial cells and fibroblast cells. VCAM-1 expression is higher in case of glomerular parietal epithelial cells, mesangial cell, tubular epithelial cell, following stimulation with cytokines. After gaining access to outside the blood vessel, neutrophil moves toward chemokine gradient and starts moving toward second tier of chemoattractant generated by pathogen byproducts localized in tissue. This directional motility of neutrophils towards pathogen occurs by signaling through G protein coupled receptors on neutrophils. Once G-protein coupled receptors binds to ligand byproducts from pathogen it gets modified into Gβγ and GTP-Gα [14]. This GTP-Gα activation modifies neutrophil polarity and changes its motility toward inclined site and now the neutrophil is called as Activated neutrophil. In activated neutrophil, there is arrangement of distinct Rho proteins at front (RAC-1) and back (Rho-A), neutrophil create actin formation that derive movements.

Rho proteins are central regulators of multiple intracellular process in immune cells. As chemokines interact with G protein coupled receptors on surface of neutrophils. G-protein coupled receptors activates lipid kinase pathway PI3 kinase activated [40]. This leads to conversion of PI [4, 5] bispo4 to PI [3, 4, 5,] tripo4 at plasma membrane. This PI [3, 4, 5] tripo4 stimulates F-actin reorganization at leading edges driving lamellipodia formation. Thus, gradient of PI [3, 4, 5] tripo4 is established at leading edges or trailing edges which promotes polarization and directional motility towards site of inflammation. There is increased accumulation of neutrophil in peritubular capillaries of outer medulla within 30mins of injury, then it transmigrates into intersitium thereby increases vascular permeability there occurs degranulation of neutrophil [21].

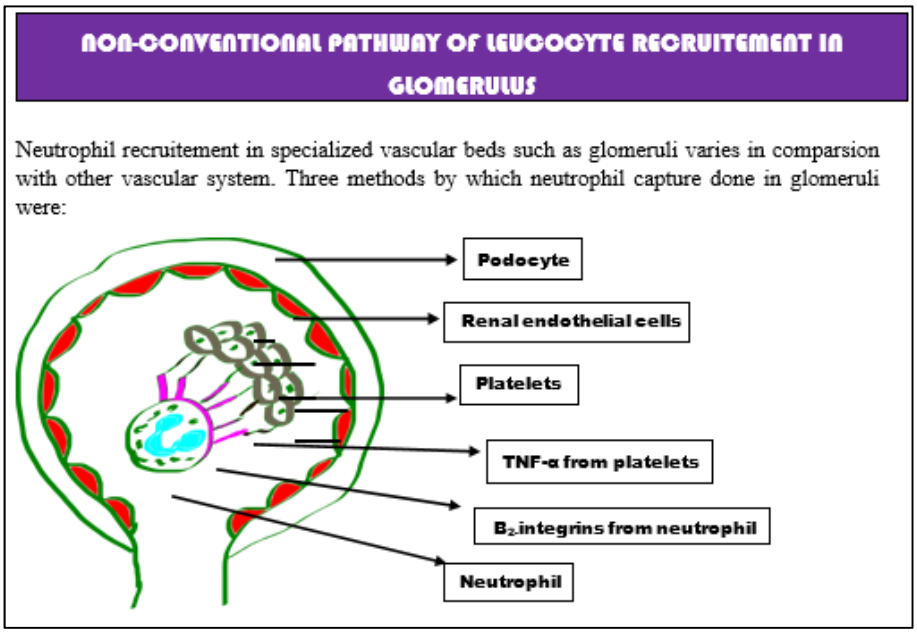

Fig 4: Secondary capture- by which platelets serve as a bridge between endothelial cells and neutrophil.

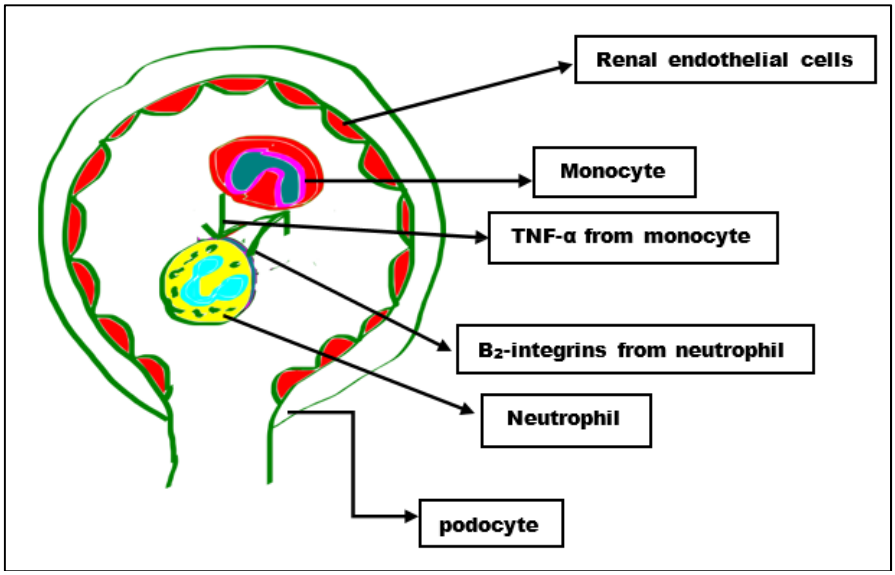

Fig 4: Increased neutrophil retention in glomeruli by direct interaction between patrolling monocyte that produces TNF-α and neutrophil that produces integrins.

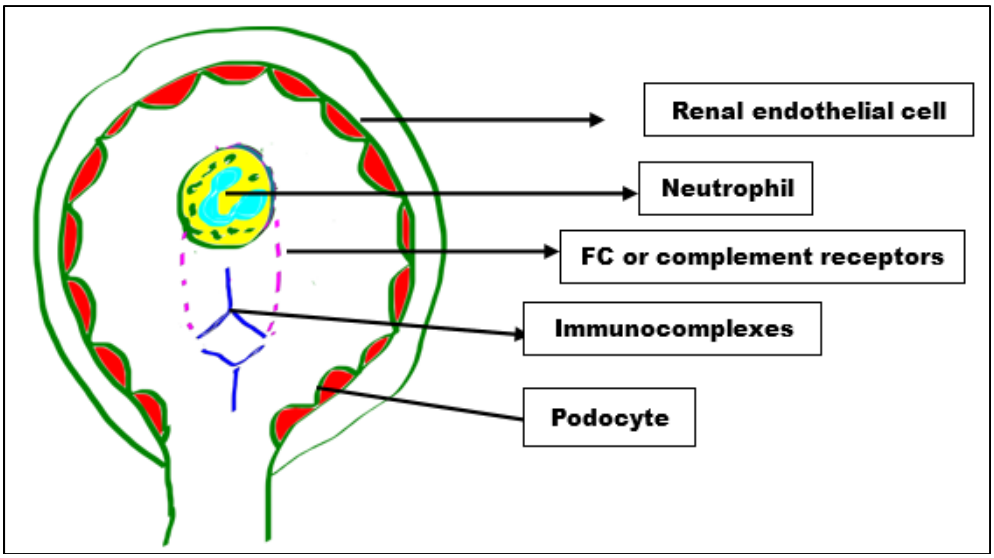

Fig 5: Third method of capture involves direct interaction of neutrophil with immune complexes through Fc or complement receptor.

Granule Mobilisation and Regulation of Exocytosis

Neutrophil upon stimulation undergoes a series of immediate changes without need for de- novo synthesis. Exocytosis or degranulation involves release of preformed mediators from granules at site of inflammation [9]. Specific steps of exocytosis involve movement of granule toward a target membrane via actin remodeling and microtubule assembly which is followed by tethering and docking through sequential action of core fusion machinery of Rab and SNARE proteins. In neutrophils, t-SNARE protein namely syntaxin 4 found exclusively on plasma membrane, whereas cognate V-SNARE, VAMP-2 present in membrane of mobilizable granules, secretory vesicles and gelatinase granules followed by granule exocytosis. Elevation of intracellular calcium known to elicit exocytosis of storage granules. Exocytosis percentage for secretory vesicles (100%), secondary granules (22%), tertiary granules (38%) and primary granules (7%) after stimulation. Fusion of azurophilic and specific granules with phagosomes creates condition for oxygen dependent and independent bactericidal activity. This NGAL is released at this stage from specific and gelatinase granules as a assembled package. Three novel proteins of specific granules contribute to function of neutrophil: this are NGAL, hcAp 18, SGP 28 [24]. NGAL named because its ability to complex with gelatinase. Insulin produced by beta-langherhans cells normally maintains healthy vasculature, they also helps in release of metalloproteinase (MMP-8 and MMP-9) from neutrophils which helps in degradation of extracellular matrix and helps out in carrying many of functions of neutrophils, whereas glucagon releases cathepsin-G from neutrophils which promote inflammation and further aggravates the condition this sought of changes occurs with metabolic changes and hormonal changes especially in case of diabetic nephropathy [17]. It has been hypothesized that NGAL participates in regulating inflammatory mediators such as FMLP, PAF, Lipopolysaccharides (LPS) and Leukotriene (B4). NGAL also exhibit chemotactic and proinflammatory properties. NGAL mediates inflammation and trys to resolute inflammation if inflammation is severe in the cells unfolded protein response occurs which trys to protect the cell. Unfolded protein response is a cellular stress response related to endoplasmic reticulum stress. This response is activated in response to accumulation of unfolded protein with response to inflammation which trys to restore normal function of cell by halting translation, to degrade misfolded proteins. Oxidative stress and ER stress occurs together in endothelial cell this endoplasmic reticulum is in close contact with mitochondria forming mitochondria associated ER membrane (MAMs) they gets activated with prolonged inflammatory response PERK receptors get activated calcium permeability increases, permeability transition pore (PTP) opens which induces release of caspase activating factor which ends up with apoptosis in case of chronic inflammation.

NGAL as a biomarker

A biomarker is renal and non renal derived molecules that report on functional status of kidney filtration and tubular injury [28]. NGAL acts as a biomarker that gets upregulated during acute kidney injury or during sepsis [32]. NGAL excreted in urine in free form or complexed with matrix metalloproteinase that degrades ECM [32]. This NGAL exerts bacteriostatic effect by modulating iron homeostasis [4]. Most of bacteria/pathogens needs iron as a mandatory survival factor to establish pathogenicity in the host. This pathogens sequestrates iron from the host by secreting siderophores [20]. This NGAL released from neutrophils couples with available extracellular iron source and forms Holo NGAL. This Holo NGAL transport iron into cell and increases systolic iron concentration. This leads to decreased availability of iron to pathogens and leads to death of organism thereby it helps out in phagocytosis [18]. Apart from its bacteriostatic effect they are also a key regulator of iron trafficking by means of forming Apo NGAL and Holo NGAL. This process is mainly mediated by binding to megalin or 24 p3R receptor on renal epithelial cells. As with increased siderophore concentration secreted by bacteria a Holo NGAL captures all siderophore along with iron molecule bind with megalin receptor gets endocytosed, releases iron inside cytosol and degrades siderophores. This released iron mediates iron dependent gene activation which leads to the formation of reactive oxygen species [34]. Hence NGAL is also known as siderocalins. Apo-NGAL reverse mediates the exocytosis of iron molecule thus NGAL acting as transport protein mediates iron trafficking [31].

Thus, NGAL mediates inflammation and gets excreted in free form in urine or complexed with metalloproteinase. With their rapid expression in early stages of renal disease, NGAL used

as acute kidney injury marker (6). NGAL plasma concentration is 100ng/ml(mice) and its levels are quantifiable. Since NGAL levels are quantifiable along with its good stability and resistance to proteases makes it a biomarker of choice for clinical use. During Acute kidney injury, NGAL was highly synthesized by cells of Thick ascending limb of loop of henle and in collecting duct only in ischemic areas of kidney [2] It has also been proved that NGAL increase in urine is mainly due to renal origin than that of extrarenal origin. In case of non-renal disease, circulating NGAL of extrarenal origin released into systemic circulation at site of inflammation by immune cells, then filtered by renal glomerulus. Most NGAL would be reabsorbed by proximal tubule which expresses megalin and remaining NGAL would be excreted in urine.

During AKI, NGAL plays a protective role by sequestrating all iron from site of injury and exports it extracellularly by forming Apo-NGAL. This leads to decreased iron thus decreasing oxidative stress and decreasing ROS formation and thereby protecting kidney [36]. In case of CKD, it excaberates the kidney disease by acting as a proinflammatory factor and acting as an effector of proliferative effects of epidermal growth factor receptor. Both this factor leads to progression to CKD [28]. Hence NGAL acts as real biomarker to detect ongoing kidney damage especially CKD stage 3 or 4 [37].

In conclusion, NGAL protects kidney during AKI by its iron trafficking effect and it damages kidney in chronic cases by its proinflammatory and proliferative effect.

Apart from its role in kidney failure, NGAL is also shown to increase in serum with all cardiomyopathies such as myocardial infarction, heart failure, atherosclerosis, any autoimmune disease [11, 12, 39]. In all this disease serum NGAL concentration was higher than urine since kidney are in functional state and trys to reabsorb all secreted NGAL and excrete very little amount in urine. Hence urinary NGAL: serum NGAL concentration always measured in order to detect NGAL of renal or non-renal origin. In renal origin, urinary NGAL was higher compared to serum and vice versa in non-renal origin. NGAL also complexes with Metalloproteinase (MMP-9) which is also known as gelatinase-B and gets elevated in endometrial cancer, hence NGAL along with MMP-9 can be used as a marker to determine various stages of cancer [16].

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