

# **More than a simple biomarker : Role of NGAL in cardiovascular and renal diseases**

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## **ABSTRACT**

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a small circulating protein that is induced in a wide variety of pathological situations, making it an interesting biomarker of various disease states. It is considered as one of the best markers of acute kidney injury, since it is rapidly released after tubular damage. However, a growing body of evidence points toward an implication of NGAL beyond its role as a biomarker. Indeed, numerous studies have demonstrated a role for NGAL in the mediation of both cardiovascular and renal diseases. In the present review, we summarize what is known about NGAL involvement in cardiovascular and renal diseases and discuss the different mechanisms underlying its pathological implications.

## **INTRODUCTION**

Neutrophil Gelatinase-Associated Lipocalin (NGAL) (also known as lipocalin-2, 24p3, siderocalin or uterocalin) is a small secreted glycoprotein of 25kDa. NGAL has been initially identified in mature neutrophil granules (1) but has since been described in many other cell types. NGAL is expressed in renal (2), endothelial (3), liver (4), and smooth muscle cells (SMCs) (5) as well as in cardiomyocytes (6), neurons (7), and in different populations of immune cells such as macrophages (5,8) and dendritic cells (9).

Several roles have been ascribed to NGAL, including iron trafficking (10), chemotactic (11,12) and bacteriostatic (13) effects as well as activities as differentiation, proliferation and growth factor (14). NGAL effects are mediated through two different receptors, 24p3R and megalin, depending on the tissue. NGAL is used as a biomarker of renal injury because it is rapidly released in response to tubular damage (15,16). However, numerous studies indicate that NGAL is more than a simple biomarker, and that it plays an important role in the pathophysiology of renal as well as cardiovascular diseases. Moreover, NGAL has been involved in various deleterious process such as inflammation and fibrosis.

### **The lipocalin family**

NGAL is part of the lipocalin protein family. This family includes many small proteins, the majority of which act as transporters, mainly for lipophilic substances. However, other roles for these proteins have been discovered, such as regulation of cell division, differentiation, cell-cell adhesion and cell survival (17). Unlike most protein families whose members are identified on the basis of similarities in their amino acid sequences, members of the lipocalin family share a common three-dimensional structure necessary for their transport function: the lipocalin fold. This structure is composed of eight anti-parallel sheets forming a

cup-shaped cavity that serve as ligand binding site (Figure 1) (17,18). Differences in amino acid sequences between members of the lipocalin family can be very large (up to 80%), allowing a wide variety of ligands within the family (17,18). However, three particular domains are very well preserved and distinguish two branches of lipocalins: the "Kernel", that have three preserved domains (such as NGAL) and the "Outliers" that only have two. (Figure 1) (17,18).

### **Structure of NGAL**

Like other members of the lipocalin family, NGAL has a three-dimensional barrel structure. Its binding site, however, has two particularities: it is polar and wide enough to bind to certain proteins (13). In addition, the presence of a cysteine residue in position 87 allows NGAL to form a disulfide bridge with a specific ligand, the Matrix Metalloproteinase 9 (MMP-9 also known as gelatinase B) (19), a protein whose enzymatic action allows the degradation of certain extracellular matrix (ECM) components and is therefore involved in tissue remodeling mechanisms (20). The binding of MMP-9 to NGAL does not modify its activity but stabilizes the protein and decreases its degradation (19). The cysteine 87 residue present in humans is absent in rodents, suggesting that NGAL/MMP-9 interaction is not possible in rodents. However, some studies suggest the opposite. Indeed, NGAL was detected in a complex with MMP-9 in the supernatant of a culture of rat SMCs (21) and co-localized with MMP-9 in mouse atheroma plaques (22). In addition, in a mouse breast cancer model, mice genetically invalidated for NGAL exhibited reduced plasma MMP-9 activity compared to Wild Type (WT) mice, illustrating a functional relationship between NGAL and MMP-9 (23).

Apart from MMP-9, NGAL is also able to interact with other ligands, in particular with certain bacterial siderophores (Figure 2).

## **Roles of NGAL**

### *Role of NGAL in iron binding and modulation*

As mentioned above, NGAL is involved in antibacterial defense through iron sequestration. Iron is an essential element for the development of bacteria, but is present in very small quantities in the body. To capture iron from the host, bacteria release proteins with high affinity for iron, i.e. siderophores. *In vitro* studies have shown that NGAL is able of binding to bacterial siderophores, thus playing a bacteriostatic role in reducing the availability of iron for bacteria (13). In addition, a study using a mouse model with genetic invalidation of NGAL (NGAL KO) showed that these mice had increased susceptibility to bacterial infections (24).

NGAL is also able to bind endogenous siderophores present in humans, i.e. the catechols. This suggests a role of NGAL in iron homeostasis, even in the absence of bacterial infection (10). When NGAL binds to an iron-coupled siderophore (holo-NGAL form), it will transport iron within the cell and thus increase the cytosolic iron concentration (Figure 3A). Conversely, when it is free (apo-NGAL form), it will allow the capture of intracellular iron and its transport to extracellular space, thus inducing a decrease in intracellular iron concentration (Figure 3B) (25). This role of NGAL in iron homeostasis could have a significant impact on pathology since iron levels are important in various deleterious mechanisms such as oxidative stress (26), inflammation (27), apoptosis (26,28) and fibrosis (28).

### *Chemotactic Role of NGAL*

NGAL has pro-inflammatory and chemotactic roles. The migration of neutrophils in culture is induced by treatment with recombinant NGAL (11,12). In addition, neutrophils from NGAL KO mice have reduced chemotactic properties and adhesion capabilities (11). In a mouse model of psoriasis (inflammatory skin disease), neutrophil infiltration into the dermis was reduced in mice treated with an anti-NGAL antibody and increased by *in vivo* treatment with recombinant NGAL (12). The recruitment of immune cells in the heart of mice subjected to an ischemia/reperfusion (I/R) episode was blunted in NGAL KO mice (29). When hearts from NGAL KO mice were transplanted into WT recipients, a significant decrease in granulocyte infiltration was observed compared to a WT to WT heart transplant (29). Numerous experiments carried out in cellular and murine cancer models have also demonstrated a role of NGAL in cell migration and invasion (30–32).

#### *Role of NGAL in differentiation, proliferation and as growth factor*

NGAL has also been shown to promote differentiation and proliferation, acting as a growth factor (14). Indeed, NGAL stimulates the proliferation and epithelial differentiation of rat embryonic kidney cells and is able to induce the tubular organization of mouse epithelial cells in culture (33). NGAL also induces the proliferation of human vascular SMCs (34) and cardiac fibroblasts (Buonafine et al. [unpublished]). The role of NGAL in cell proliferation has also been demonstrated in gastric and thyroid cancer models (35,36). NGAL participates to the epithelial-mesenchymal transition (EMT) *in vivo* in a pulmonary adenocarcinoma model in mice (37) and *in vitro* in prostate (30) and breast cancer cells (38). In these models, NGAL promoted the motility, invasiveness and metastatic capacities of cancer cells.

#### **NGAL receptors**

### *24p3 Receptor*

The 24p3 receptor (24p3R) is one of the two receptors currently described for NGAL. 24p3R is an endocytic receptor with a strong affinity for NGAL which allows it to penetrate inside the cells. It participates to the control of iron homeostasis by allowing NGAL to enter the cells, thus modulating the amount of intracellular iron. The expression of this receptor has been identified in different tissues and notably in the heart (25). Under inflammatory conditions, 24p3R is expressed in the whole cardiac tissue and, in particular, on the surface of cardiomyocytes (39). In addition, 24p3R is expressed in the renal distal nephron (40) and is involved in albumin endocytosis and activation of pro-inflammatory and pro-fibrotic signaling pathways of NF- $\kappa$ B and TGF- $\beta$  (41). 24p3R expression was found to be increased on the surface of neutrophils of patients with psoriasis (12). Finally, the use of an anti-24p3R siRNA has also highlighted the important role of this receptor in the activation of neutrophils by NGAL in culture (12).

### *Megalin*

The other known receptor for NGAL is megalin (or low-density lipoprotein receptor-related protein 2, LRP2). Megalin is a multi-ligand endocytic receptor which is expressed in various epithelia and in particular in epithelia with high absorptive capacity such as the epithelium of the renal tubule, ileum, or choroid plexus in the brain (42). Megalin has also been detected in cardiomyocytes cultured in vitro (43) as well as in different types of immune cells such as T-cells, B-cells, granulocytes and monocytes/macrophages (44). Megalin belongs to the family of low-density lipoprotein receptors (45) and has been shown to bind to various lipocalins (18,46). However, its affinity for NGAL is higher than for other lipocalins (47). The pathophysiological role of the NGAL-megalin complex is still not well described.

## **Involvement of NGAL in renal pathologies**

NGAL has been described as an acute renal lesion biomarker because it is rapidly released in response to tubular damage (15,16). NGAL is a secreted protein and can be quantified in plasma or serum. In healthy humans, plasma concentration of NGAL is approximately 70 ng/mL (48) and about 100 ng/mL in mice (8,49). NGAL levels analysis is also possible in urine (50). These elements, combined with its good stability and resistance to proteases, make it a biomarker of choice for clinical use (15,16). Creatinine plasma levels are commonly used to evaluate renal function. However, an ever-growing number of studies describe NGAL as a better marker of acute kidney injury (AKI).

### *NGAL in AKI*

In animals, ischemia/reperfusion (I/R) induces a massive increase in renal NGAL levels within 3 hours after ischemia, while the increase in serum creatinine is still mild (51). In addition, serum creatinine concentration increases after severe bilateral ischemia but remains unchanged after mild bilateral or unilateral ischemia (51). In AKI, NGAL levels are increased up to 300 times in blood (0.1 to 30  $\mu\text{g/mL}$ ) and 1000 times in urine (0.04 to 40  $\text{mg/mL}$ ) (49,51).

In patients with AKI, the relative level of serum NGAL was correlated with the severity of renal damage and high levels of serum NGAL were associated with an increased risk of mortality (52). In addition, urinary and serum NGAL concentrations have been described as a sensitive, specific and highly predictive markers of AKI after cardiac surgery (50). Several studies have also suggested NGAL as a marker of renal damage in broader pathological contexts than renal ischemia such as glomerulonephritis and IgA nephropathy (53,54).

Using a mouse model expressing a bioluminescent reporter of NGAL expression, Paragas *et al.* have studied the origin of NGAL during AKI. They determined that during I/R, NGAL was synthesized by cells of the thick ascending limb of Henle's loop and of the collecting duct and only in ischemic areas of the kidney (55). In addition, by performing kidney transplants between WT and NGAL KO mice, Paragas *et al.* demonstrated that the increase in urinary NGAL concentration during renal ischemia was mainly due to the release of NGAL originating in the kidney and that the contribution of extra-renal NGAL to urinary NGAL was small (55).

Another suggested source of urinary NGAL, especially in non-renal diseases, could be circulating NGAL from extra renal origin, being released in the systemic circulation at sites of inflammation, notably by immune cells (56), and then filtered by the renal glomerulus. The majority of NGAL would then be reabsorbed by the proximal tubule, which expresses megalin, and the remaining NGAL would be excreted in the urine. This last point is illustrated, for example, by a study showing that mice deficient in megalin show a leakage of NGAL in the urine (47).

Beyond its role as a biomarker, NGAL is actively involved in the mechanisms underlying kidney damages. NGAL has been shown to play a protective role in AKI after an episode of I/R (49,57). During an I/R episode, the release of large amounts of iron at the time of ischemia promotes oxidative stress and induction of tissue damage. In addition, the subsequent reperfusion further increases the amount of iron, exacerbating the damage caused by oxidative stress (58,59). In animal models, the use of iron-binding protein (60,61), including the injection of recombinant NGAL (49,57), has been shown to limit early damage caused by I/R or rejection of the graft during renal transplantation (62). A study also showed that intravenous injection of macrophages over-expressing the anti-inflammatory cytokine IL-

10 was able to protect against renal ischemia in rats and improve cell regeneration and tissue repair through the induction of NGAL (63). When rats were treated with an anti-NGAL antibody, the protective role of the adoptive transfer of macrophages over-expressing IL10 was lost (63), underlying a crucial role of NGAL. More recently, a study showed that the infusion of macrophages overexpressing NGAL could improve renal fibrosis in a unilateral ureteral obstruction model in mice (64).

#### *NGAL in CKD*

In contrast to its protective effect in AKI, NGAL is described to be harmful in the long term. NGAL is considered as a pro-inflammatory factor that promotes the progression toward CKD (65,66). A high level of urinary NGAL was associated with a higher risk of CKD (66,67). In CKD patients (66,68) or in mouse models of CKD (65,66), NGAL levels were increased and correlated with the severity of renal injury.

The role of NGAL in CKD has been investigated in animal models, particularly using NGAL KO mice. In an anti-glomerular basement membrane antibody-induced glomerulonephritis, NGAL gene invalidation protected mice from proteinuria and tubular lesions while the addition of recombinant NGAL exacerbated kidney disease and decreased survival (65). Similarly, in a subtotal nephrectomy-induced CKD model, NGAL KO mice exhibited less apoptosis, less renal lesions (glomerulosclerosis, tubular atrophy, interstitial fibrosis, immune infiltration), less proteinuria and better renal function than WT mice (66). Interestingly, this study also showed that NGAL was an effector of the proliferative effects of EGFR (Epidermal Growth Factor Receptor) (66) which is known to play an important role in the progression of CKD (69).

In conclusion, the role of NGAL in kidney disease seems to depend on the associated pathological mechanisms: after an acute kidney injury, NGAL plays a protective role, notably

via its iron-modulating effects, whereas in a more chronic context, its pro-inflammatory and proliferative effects make it a harmful actor.

### **Involvement of NGAL in cardiovascular diseases**

Besides its role as a biomarker of kidney lesion and its implication in the pathophysiology of renal diseases, NGAL has also been shown to be involved in the development of cardiovascular (CV) diseases.

#### *NGAL in myocardial infarction and heart failure*

Several studies have documented elevated circulating NGAL levels in patients with CV disease (70,71). In patients with acute myocardial infarction (MI) or chronic heart failure (HF), serum NGAL levels are higher than in healthy subjects (70,72). Plasma NGAL levels are higher in MI patients than in patients with stable coronary heart disease (73). Some studies suggest that NGAL may have prognostic value in HF patients because high levels of plasma or urinary NGAL are associated with more renal complications (70,72,74) or mortality (75–77). In a 10-year follow-up study of healthy subjects, a higher basal level of NGAL was associated with an increase in the proportion of adverse cardiac events and overall mortality from all causes (78). These high levels of NGAL may be explained in part by the renal failure observed in a large number of HF patients (79). However, several studies have showed that NGAL was a predictor of CV incident even in the absence of renal dysfunction (80–82). Circulating NGAL levels have also been described as predictors of CV complications in patients with CKD (83,84).

In animals, NGAL production is increased in the heart and aorta after MI (22,72). NGAL production is also increased in isolated rat cardiomyocytes in response to stimulation by different pro-inflammatory molecules such as endothelin 1, IL-1 and TNF- $\alpha$  (72). In a recent study from our laboratory, we demonstrated an important role of NGAL in the pathophysiological mechanisms of MI. NGAL KO mice showed decreased cardiac fibrosis and inflammation as well as preserved cardiac function 3 months after MI (85,86). A recent study from Sung *et al.* using a similar MI model showed that NGAL KO mice exhibited an increase in autophagy associated with decreased apoptosis and preserved cardiac function after infarction (87).

#### *NGAL in atherosclerosis*

In arteries of atherosclerotic patients, NGAL expression was observed in endothelial cells, SMCs and macrophages and was detected at a higher rate in patients with symptomatic carotid stenosis compared to asymptomatic patients (5). The level of circulating NGAL was also associated with plaque vulnerability (88). In a mouse atherosclerosis model, NGAL expression was co-localized with macrophages and MMP-9 in the atherosclerotic plaques, suggesting a possible role of NGAL in MMP-9-mediated remodeling (22).

#### *NGAL in cardiovascular ischemia*

The role of NGAL has also been shown in the cardiac lesions induced by I/R (89,90). After an episode of I/R, in a model of isolated-perfused heart, NGAL KO heart exhibited a reduced infarct size and better cardiac contractile function than the WT heart (90). In addition, increasing NGAL circulating levels of NGAL KO mice using an NGAL encoding adenovirus treatment resulted in impaired cardiac functional recovery and decreased mitochondrial function of the isolated-perfused hearts (90). In a mouse model of I/R after heart

transplantation, the use of anti-NGAL antibodies reduced the infiltration of macrophages and neutrophils into the ischemic zone, suppressed the M1 polarization of macrophages, and blunted the I/R-induced cardiac lesions (89).

In a rat model of cerebrovascular ischemia, a large increase of NGAL and MMP-9 was found in the intima of the common carotid artery (21). In this model, blocking the NF- $\kappa$ B pathway *in vivo* led to an almost total suppression of NGAL and MMP-9 expression, suggesting a central role of NF- $\kappa$ B signaling in NGAL and MMP-9 transcriptional regulation in this context (21). *In vitro*, expression of NGAL in cultured SMCs was also induced in an NF- $\kappa$ B dependent manner by treatment with IL-1 $\beta$  (21).

#### *NGAL in abdominal aortic aneurysm*

Biopsy analysis of patients with abdominal aortic aneurysm (AAA) identified the expression of the NGAL/MMP-9 complex in the vascular wall and in the thrombus present in the blood vessel lumen (91). Neutrophils were the major source of NGAL expression in this context. In an AAA model in mice, the absence of NGAL (in NGAL KO mice) or its blockade (with anti-NGAL antibodies) had the same protective effects against AAA development, with a decrease in neutrophil infiltration and MMP activity (92).

#### *NGAL in cardiometabolic disorders*

In obesity in humans and animals, circulating NGAL levels are increased and correlated with increases in blood pressure and insulin resistance (93). A recent study from our laboratory also reported an increase in plasma levels of the NGAL/MMP-9 complex in a cohort of obese patients correlated with an increase in circulating fibrosis markers (94).

In animal models of obesity, NGAL KO mice were protected against hypertension (95), inflammation (95,96) as well as endothelial (97) and cardiometabolic dysfunctions

(90,96,97) induced by a high fat diet. Song *et al.* reported that the deamidation of NGAL (by linoleic acid in particular) improved its stability and promoted its accumulation (95). The combined administration of recombinant NGAL and linoleic acid in WT mice promoted oxidative stress, endothelial dysfunction, inflammation and hypertension (95).

#### *NGAL and iron metabolism in cardiomyopathies*

NGAL participated to iron transport by binding a siderophore and can increase or decrease the amount of intracellular iron, thus participating in the maintenance of iron homeostasis. Both iron overload and iron deficiency have been linked to cardiomyopathies: iron overload has been associated with increased oxidative stress and iron deficiency with mitochondrial dysfunction, impaired cardiac function (98), hyper-coagulation and oxidative stress related to anemia (99). The involvement of NGAL in iron homeostasis could therefore play an important role in the development of some cardiomyopathies. Indeed, NGAL has also been shown to participate to cardiomyocyte apoptosis resulting from intracellular iron accumulation (100). The apoptosis of cardiomyocytes could therefore influence the remodeling process involved in the development of certain cardiac pathologies, underlying a potential implication of NGAL in this process.

#### **Involvement of NGAL in inflammatory mechanisms**

##### *NGAL and pro-inflammatory cytokines expression*

In heart failure and various other inflammatory pathologies, serum or plasma levels of NGAL have been shown to correlate with those of other inflammatory markers such as TNF- $\alpha$ , CRP, IL-6 or leukocytes number (101,102).

NGAL expression can be induced by various pro-inflammatory stimuli, such as LPS (103), IL-1 $\beta$  (104), IL-6 (3), IL-17 (105), IFN- $\gamma$  (106) and TNF- $\alpha$  (72) depending on cell type. Furthermore, NGAL expression is positively regulated by the activation of the NF- $\kappa$ B pathway (21) which is known for its role in pro-inflammatory mechanisms. Conversely, NGAL is able to activate the NF- $\kappa$ B pathway (107,108) and to induce the expression of diverse pro-inflammatory molecules such as IL-8 (5,12), IL-6 (5,12), IL1- $\alpha$  (12), TNF- $\alpha$  (12) and MCP-1 (5). Finally, it has been shown that NGAL was involved in the polarization of macrophages towards the pro-inflammatory phenotype M1, *in vitro* and *in vivo* (89,109).

Altogether these data suggest the existence of a vicious circle in which NGAL is overexpressed in inflammatory conditions and capable, in turn, to potentiate inflammation by inducing the expression of pro-inflammatory mediators (Figure 4).

#### *NGAL and inflammatory diseases*

NGAL is proposed to be involved in chronic inflammation and autoimmune diseases. Urinary levels of NGAL were increased in patients with lupus nephropathy and were associated with the severity of the disease (110). In a rat autoimmune myocarditis model, NGAL was strongly expressed in cardiomyocytes, vascular SMCs, fibroblasts and neutrophils (39). In addition, increased NGAL levels were particularly important during the active phase of myocarditis and closely followed cardiac and plasma IL-1 $\beta$  levels (39). In an acute antibody-induced skin inflammation model, NGAL KO mice showed a 50% reduction in inflammation associated with reduced immune infiltration compared to WT mice (111). The

same benefit was found by injecting WT mice with anti-NGAL antibodies while inflammation was restored by treatment with recombinant NGAL (111).

#### *NGAL expression by immune cells*

NGAL expression was first described in neutrophils (1) and later observed in other immune cell types such as macrophages (5,8) and dendritic cells (9). Recent studies suggest that the expression of NGAL by these immune cell types could be important in the regulation of inflammatory processes. It has been shown, for instance, that NGAL secreted by dendritic cells was involved in the activation and polarization of T-cells toward a pro-inflammatory phenotype (9). Gilet *et al.* showed that aldosterone was able to induce the production of NGAL by human neutrophils in complex with MMP-9 (112). Aigner *et al.* identified neutrophils as the primary source of NGAL released at the time of immune infiltration in the mouse heart after an episode of I/R (29).

A recent study from our laboratory, using mice depleted from NGAL in their immune cells only (after bone marrow transplantation), allowed us to demonstrate the pivotal role of NGAL produced by immune cells in the cardiac and renal deleterious effects of a mineralocorticoid challenge (Buonafine *et al.* [unpublished]). In WT mice, this challenge induced systemic inflammation and an induction of NGAL expression by macrophages, dendritic cells, and peripheral blood mononuclear cells (PBMCs). The depletion of NGAL from immune cells protected the mice against cardiac and renal remodeling as well as inflammation induced by mineralocorticoid excess (Buonafine *et al.* [unpublished]). Of note, mice depleted of NGAL in their immune cells presented lower levels of cardiac NGAL compared to control mice, revealing immune cells as a major source of NGAL in the heart (Buonafine *et al.* [unpublished]).

### **Involvement of NGAL in hypertensive mechanisms**

In patients with essential hypertension, plasma NGAL level were higher than in healthy subjects and correlated with blood pressure (113). In clinical studies, polymorphisms in the promoter of NGAL have been associated with changes in blood pressure (114).

The role of NGAL in hypertensive mechanisms was also demonstrated in animal models of obesity. NGAL KO mice were protected against hypertension, inflammation and cardiometabolic dysfunction induced by a high fat diet (95–97). The direct role of NGAL in blood pressure control was further demonstrated by the combined administration of recombinant NGAL and linoleic acid in mice, which induced an increase in mouse blood pressure (95). Recently, a study from our laboratory demonstrated the crucial role of NGAL in the setting of aldosterone-mediated hypertension. Indeed, the global genetic inactivation of NGAL in mice prevented the increase in blood pressure induced by a mineralocorticoid challenge (94).

### **Involvement of NGAL in pro-fibrotic mechanisms**

The role of NGAL in pro-fibrotic mechanisms was first suggested by the identification of its binding to MMP-9, a protein involved in extracellular matrix remodeling (19). Our laboratory demonstrated recently the crucial role of NGAL in the pro-fibrotic effects of a mineralocorticoid challenge (94) and in a model of MI (85).

The mechanisms by which NGAL induces fibrosis are probably diverse. NGAL binding to MMP-9 may be one, although the existence of NGAL/MMP-9 in rodents remains controversial. It is also possible that NGAL binds to other molecules able of modulating fibrosis. For example, NGAL binds and negatively regulates the activity of HGF (Hepatocyte Growth Factor), a peptide known for its anti-fibrotic properties (115). We recently showed a direct pro-fibrotic role of NGAL in human cardiac fibroblasts ((85); [Buonafine et al.](#)

[unpublished]). The treatment of fibroblasts with aldosterone induced the expression of NGAL and collagen I. However, inhibition of NGAL expression by siRNA resulted in the loss of induction of collagen I expression by aldosterone. This suggests that NGAL could act as a mediator of the pro-fibrotic effects of aldosterone *in vivo* (85).

In addition, our data indicate that NGAL has an effect on the proliferation of fibroblasts *in vitro* (Buonafine et al. [unpublished]). This proliferative effect of NGAL was also described *in vivo* in renal I/R models in which recombinant NGAL injection induced tubular cell proliferation (49,57) while NGAL KO mice had reduced tubular proliferation in a mouse model of CKD (66). Given the key role of proliferation (of myofibroblastes in particular) in the mechanisms of remodeling and fibrosis (116), the proliferative effects of NGAL could also be implicated in pathological organ remodeling.

### **Signaling pathways involved in the pathological effects of NGAL**

#### *Erk1/2 signaling pathway*

Another signaling pathway modulated by NGAL is the ERK1/2 pathway. This signaling pathway is known for its involvement in cell proliferation and cell death processes (117). Aldosterone has been shown to induce NGAL/MMP-9 in human neutrophils in culture, and this effect was prevented by the use of an ERK1/2 inhibitor (112). The role of the ERK1/2 pathway in the induction of NGAL has also been described in intestinal epithelial cells in response to a bacterial toxin (118). It is interesting to note that expression of the NGAL receptor 24p3R was induced by IL-1 $\beta$  in human mesangial cells in culture and also involved activation of the ERK1/2 signaling pathway (119). Conversely, several studies have shown that NGAL is able to activate the ERK1/2 signaling pathway and that this pathway is involved in the cellular effects of NGAL. Treatment of cultured epithelial cells with NGAL

induced activation of the ERK1/2 pathway and induced cell migration, which was lost through inhibition of this signaling pathway (33). Similarly, the treatment of human neutrophils in culture with recombinant NGAL stimulated cell migration and pro-inflammatory cytokine expression via the 24p3R receptor and induction of the ERK1/2 signaling pathway (12). In models of esophageal (31) or prostate cancer cells (120), overexpression of NGAL or treatment with recombinant NGAL activated the ERK1/2 pathway and blocking this pathway blocked the migration and invasive properties of the cells promoted by NGAL.

#### *NFκB signaling pathway*

A large number of studies have shown that the NF-κB pathway controls the expression of NGAL (4,21,35,36). Conversely, some studies have shown that NGAL itself is capable of activating the NF-κB pathway (37,107,108), which is of primary importance given the major involvement of this pathway in inflammatory and fibrotic mechanisms (121,122). In a cell model with NGAL overexpression, Wang *et al.* showed, for example, that NGAL activated the NF-κB pathway via increased levels of intracellular iron and reactive oxygen species (108). Consistent with this, we recently showed that NGAL activated the NF-κB pathway in cardiac cells, *in vivo* and *in vitro* (85). The treatment of cardiac fibroblasts with recombinant NGAL induced NF-κB activation while blocking the NF-κB pathway prevented NGAL-induced production of collagen I (85). The NF-κB pathway was activated in WT mice seven days after MI, but this was not the case in NGAL KO mice, demonstrating the importance of NGAL in activating this pathway *in vivo* (85). These findings highlight the importance of the NF-κB pathway in mediating the direct pro-fibrotic effects of NGAL.



## CONCLUSION

In conclusion, NGAL appears to be an important mediator of cardiac and renal diseases through its role in hypertensive, inflammatory and fibrotic processes. High levels of NGAL are reported in a wide variety of pathological situations, both in animals and in patients, and NGAL blockade using neutralizing antibodies or genetic inactivation is beneficial in animal models of cardiac or renal injuries. The role of NGAL in inflammation is of particular importance since NGAL is part of a pro-inflammatory amplification loop. NGAL is induced in inflammatory situations and is able to amplify inflammation, by the production of pro-inflammatory cytokines, the activation of pro-inflammatory pathways as well as the polarization of immune cells toward pro-inflammatory phenotypes. This pro-inflammatory role of NGAL could be important in the mediation of its hypertensive and fibrotic effects, since the interconnection of these pathological mechanisms is now well described (123–125). Finally, NGAL production and secretion by immune cells themselves seem to play an important role in some cardiorenal pathological situations. The role of NGAL produced by specific immune cell populations should thus be further studied.

Given the importance of its pathological implications, NGAL could represent an interesting therapeutic target in CV and renal diseases and beyond, especially in pathologies involving inflammatory mechanisms.

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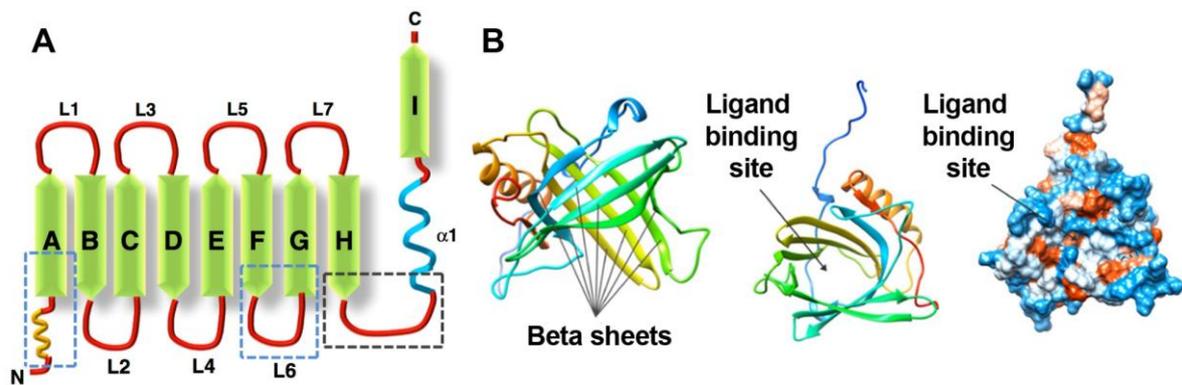
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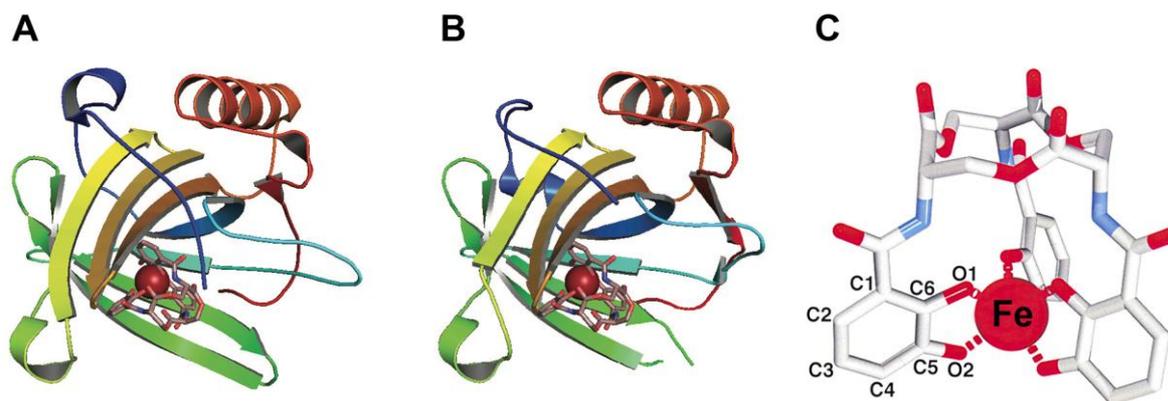
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## FIGURES



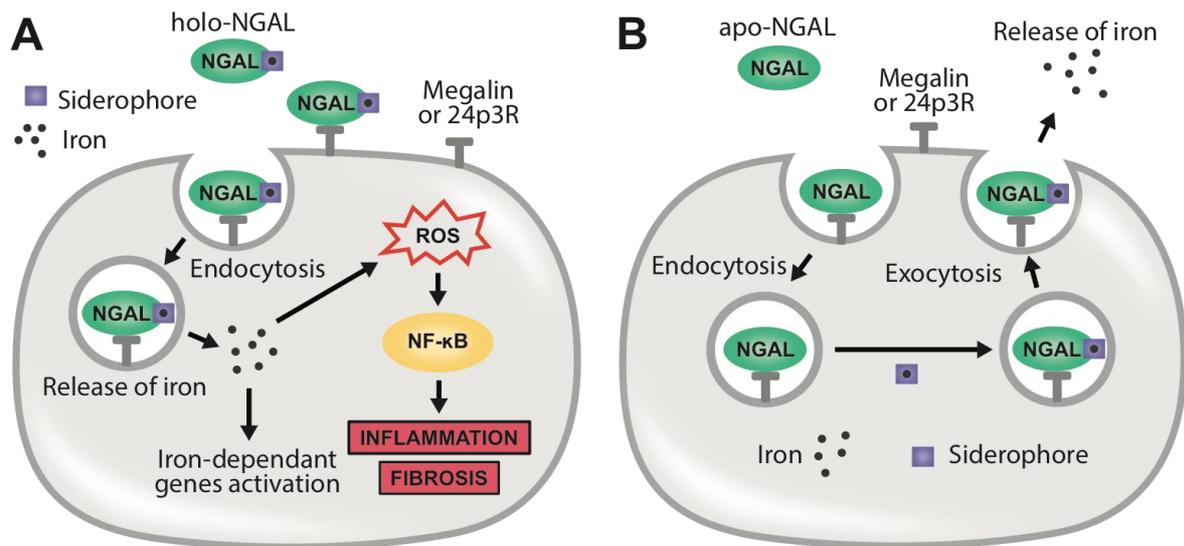
**Figure 1 – Schematic structure of lipocalins**

**(A) Schematic representation of the lipocalin fold.** The areas framed in blue correspond to the domains structurally preserved within the lipocalin family and the region framed in black corresponds to a domain showing significant conservation in amino acid sequence. Adapted from Chakraborty *et al.* 2012 (17). **(B) Tridimensional structure of lipocalins.** Adapted from Candido *et al.* 2014 (126).



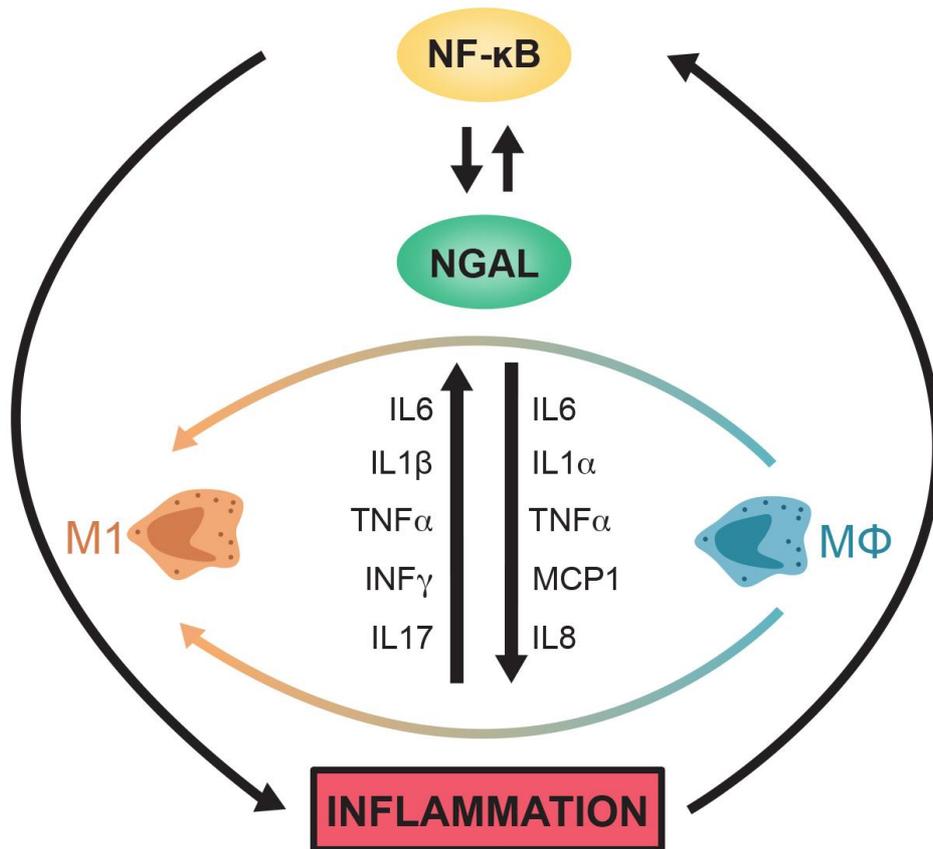
**Figure 2 – Three-dimensional structure of the NGAL-siderophore-iron complex**

Three-dimensional representation of NGAL in humans (A) and mice (B). The red sphere in the center corresponds to an iron atom and the surrounding structure corresponds to a siderophore. Adapted from Xiao *et al.* 2017 (127). (C) **Representation of a siderophore-iron complex to which NGAL can bind.** The atoms are identified by their color: C in grey, N in blue, O in red. The red sphere corresponds to the iron atom in the center of the siderophore. Adapted from Goetz *et al.* 2002 (13).



**Figure 3 – Role of NGAL in iron trafficking**

**(A) Importation of iron into the cell.** NGAL bound to an iron-coupled siderophore (holo-NGAL) is endocytosed in the cell thanks to its membrane receptor (Megalin or 24p3R) thus allowing the release of iron into the cytoplasm. The increase in cytoplasmic iron levels will induce the expression of iron-dependent genes and promote oxidative stress which, in turn, activates the NF-κB pathway, known to be involved in inflammatory and fibrotic mechanisms. ROS: Reactive Oxygen Species. **(B) Exportation of the iron out of the cell.** Free NGAL (apo-NGAL) is endocytosed into the cell where it binds to an iron-coupled siderophore. It is then exocytosed out of the cell, thus allowing the release of iron in the extracellular medium and the diminution of the intracellular iron stock.



**Figure 4 – Implication of NGAL in a pro-inflammatory amplification loop**

NGAL expression is induced by several pro-inflammatory cytokines as well as NF-κB pathway activation. Conversely, NGAL has pro-inflammatory effects, inducing the expression of various pro-inflammatory cytokines and activation of the NF-κB pathway. NGAL is also involved in the polarization of macrophages towards the pro-inflammatory phenotype M1.

MΦ : Macrophage, M1 : Pro-inflammatory M1 macrophage