Neutrophil Gelatinase Associated Lipocalin (NGAL) as a Biomarker of Painful Vaso-Occlusive Crisis in Sickle Cell Disease (SCD)

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Abstract
Chronic inflammation and hypercoagulable state contribute significantly to the occurrence of vaso-occlusive complication of sickle cell diseases (SCD). The pathogenesis determines changes in the levels of pro-inflammatory mediators such as cytokines, endothelial adhesion molecules, elevated markers of thrombin generation and group of new biochemical markers including NGAL. The aim of the present study was to evaluate levels of neutrophil gelatinase associated lipocalin (NGAL) in SCD patients during painful crises and steady state of illness. This work included 43 patients with SCD in painful crises that was severe to justify hospital admission. Nineteen samples were collected among the cases when patients came to the clinic for follow up visit in steady state. Control group included fourteen healthy ethnically matched individuals. NGAL levels were quantitated by ELISA. Other hematological and biochemical parameters such as HG, RBC, WBC, bilirubin, CRP, LDH were also determined.

The results revealed that NGAL concentrations were highly significantly elevated in sickle cell painful crises compared with steady state SCD (p<0.0001) and when compared with healthy controls. These results also showed the NGAL levels correlate with WBC, CRP and LDH.

This work represents initial step to determine NGAL role as a biomarker involved in the inflammatory and immune modulatory functions reported in the pathogenesis of painful episodes in SCD and may be a valuable predictor marker in the progress to severe attaches (e.g. acute chest syndrome) or resolution of acute SCD crisis.

Key Words: NGAL – SCD – Biomarker of painful – Vaso-occlusive crisis.

Introduction
SCD is an ongoing chronic hemolysis punctuated by acute recurrent painful vascular occlusion crisis that represent the most common clinical manifestation of the disease [1]. Vascular occlusion can affect any organ of the body [2] and results in variable degree of damage in different organs in the majority of SCD patients [3]. These features of SCD have a wide range of severity, disease progression and clinical improvement. So, it is required to develop reliable biomarkers to follow-up these changes [3].

Neutrophil gelatinase associated lipocalin (NGAL) is a glycoprotein found in the specific granules of the human neutrophil. NGAL was shown to exist as a 25kDa monomer, 46kDa homodimer and in a covalent complex with neutrophil gelatinase [4]. NGAL is a member of the lipocalin family that consists of tertiary structure having one alpha helix and eight stranded beta barrel surrounding hydrophobic core. The hydrophobic core is a pocket that enables the lipocalins to bind small lipophilic substances [5]. Lipocalins may bind lipophilic inflammatory mediators like platelet activating factor, leukotriene B4 and lipopolysacchride. So, NGAL may have important immune modulatory functions [6,7]. NGAL was isolated by northern blotting of many tissues like brain, lung, heart, kidney, liver, blood and bone marrow cells [48]. In SCD, cell necrosis could happen secondary to repetitive episodes of ischemical hypoxemia and reperfusion, chronic low grade inflammation with acute exacerbation and tissue injury. In this study we evaluated NGAL in plasma of SCD patients during vaso-occlusive crisis compared to stable SCD patients and those ethnically matched controls.

Patients, Material and Methods
This study included two groups:
1- 43 patients with SCD.
2- 14 healthy ethnically matched control.

Those patients were evaluated during their admission and clinic visits in one major hospital (Mouwasat Group) in the eastern province of Saudi Arabia. All patients accepted samples collection and participation in this research.
Blood samples were collected over an eight month period (Feb 2006-Oct 2006). Samples were collected from concerning patients. Nineteen were in steady state who visited the outpatient clinic for follow-up. Forty three were admitted into hospital in acute pain. The painful crisis was considered as an episode of acute pain in the limbs and/or abdomen, chest and back resulting in hospitalization and not related to any medical problem other than SCD. Nineteen patients were studied in both crisis and steady state. Complete work-up including biochemical and haematological parameters were performed. Control subjects had NGAL determination with biochemical and haematological evaluation.

Sample preparation and quantitation of NGAL:

NGAL was quantitated by ELISA, as described previously by Kjeldson et al. Samples were stored in temperature 80º to avoid the decline of NGAL related to storage in –20º and long time storage was avoided. NGAL concentrations were determined by an MCP-1 alpha ELISA purchased from Bio-Source International Inc. (Camarillo, California).

Data analysis:

A mean value of all the steady-state samples were calculated for each patient and used in comparison with the single point crisis and healthy control sample values. Normality testing of NGAL concentration data found that the raw data did not follow a Gaussian distribution but the data were normal after log transformation. Log-transmitted values were used in all analyses but untransformed values were used for reporting results. Comparisons between groups (SCD steady-state, acute painful crisis and healthy control) were carried out using a Student’s t-test; p-values <0.001 were considered significant. Pearson’s correlation tests were performed between NGAL values and the following standard biochemical markers; haemoglobin, red blood cell count (RBC), white blood cell count (WBC), serum bilirubin levels, C-reactive protein (CRP) and lactate dehydrogenase (LDH). The results were considered significant when p<0.05. Data analyses were performed using GraphPad Prism (version 3.0) from GraphPad Software Inc., San Diego, CA, USA.

Results

The median serum NGAL concentrations in healthy controls were 240.6ng/ml (range: 113.6-685.4ng/ml). Nineteen patients during steady state revealed median serum NGAL value of 486.5ng/ml (range: 180.3-866.5ng/ml) and SCD patients in acute painful crisis revealed NGAL level ranging between 1200.4ng/ml to 10,000.5ng/ml with median value of 1600.3ng/ml. The log-transformed data were analysed unpaired Student’s t-test, with all sickle cell patients analysed as one group in the first instance. NGAL concentrations were highly significantly elevated in sickle cell painful crisis compared with steady state SCD (p<0.0001) and sickle cell crisis when compared with healthy controls (p<0.0001). NGAL values were not significantly different when the levels of healthy control subjects compared with patients in steady state.

The sickle cell patients were analysed further according to Hbss and Hbs/thalessemia (specially patients in the eastern province of Saudi Arabia have a good percentage of HbF ranging from 3 to 30 percent), there was no significant differences between these groups, which could be explained by small sample size. There was a wide range in NGAL concentration with overlap between steady state and crisis.

Circulating NGAL was not affected by age. Mean ages for the healthy control subject group and the sickle cell patient group were compared and found to be significantly different by t-test (p<0.01, 95%). Confidence interval (CI 95% = 1.245, 6.927). No correlation was found between age and NGAL levels for either control group or sickle cell patients.

Circulating NGAL levels correlate with WBC, CRP and LDH. Mean steady state NGAL levels for each patient were compared with mean values for the following markers of inflammation and hemolysis; WBC, CRP, LDH, RBC and haemoglobin levels.

Table (1): Descriptive data for SCD study cases and healthy controls.

<table>
<thead>
<tr>
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<th>Sickle cell patient</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>43</td>
<td>19</td>
</tr>
<tr>
<td>Male: Female</td>
<td>28/15</td>
<td>12/7</td>
</tr>
<tr>
<td>Age: Median (range)</td>
<td>27 (13-58)</td>
<td>35 (18-52)</td>
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<tr>
<th>NGAL ng/mL</th>
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<tr>
<td>Steady State</td>
<td>486.5 (180.3-866.5)</td>
<td>240.6 (113.6-685.4)</td>
</tr>
<tr>
<td>Crisis State</td>
<td>1600.3 (1200.4-10000.5)</td>
<td></td>
</tr>
<tr>
<td>WBC (x 10^9/L)</td>
<td>9.3 (3.8-27.2)</td>
<td>4.5 (2.9-7.6)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>56 (12-116.5)</td>
<td>44 (2-78)</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>86 (49-116)</td>
<td>14.1 (11-15.4)</td>
</tr>
<tr>
<td>RBC (x 10^12/L)</td>
<td>2.2 (1.4-4.8)</td>
<td>4.8 (3.7-5.4)</td>
</tr>
<tr>
<td>LDH (u/L)</td>
<td>385 (110-1800)</td>
<td>167 (138-243)</td>
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Pearson’s correlation tests showed a significant correlation between NGAL levels and CRP for sickle cell disease patients ($r=0.285$, $p<0.005$, CI: 95%=0.09, 0.42), significant correlation was demonstrated between NGAL levels and WBC for SCD ($r=0.218$, $p<0.05$, CI: 95%=0.01, 0.35).

**Discussion**

NGAL (neutrophil gelatinase associated lipocalin) belongs to a family of small proteins, NGAL is engaged in the transmembrane transportation of lipophylic substances [11]. NGAL was originally isolated from specific granules of neutrophils, it was later found to be located in bone marrow cells, lung, bronchial, colon epithelial cells and body fluids. NGAL expression considerably augments during the occurrence of inflammation and some cancers. Immune modulation was suggested to be the main function of NGAL as well as its bacteriostatic effect [11,12]. Acute painful crisis is the first leading cause of hospitalization in patients with SCD. Acute chest syndrome accounts up to 25% of deaths associated with the disease [13].

This study showed NGAL levels to be significantly higher in the plasma of SCD patients during acute painful crisis, compared with levels in patients during steady state. NGAL levels were not significantly different in patients with SCD who have steady state when compared with healthy controls. We propose that NGAL elevated levels during acute painful crisis in SCD reflects the acute tissue damage and inflammation associated with vaso-occlusion. This possibility is supported by the positive correlation between NGAL levels and other biochemical markers of inflammation, such as WBC counts and CRP in steady state [14,15].

Our results agree with Pierre-Louis et al. [15] who determined elevated levels of urinary NGAL and endothelin-1 as markers of renal damage in sickle cell disease [15]. Thrombogenesis has been implicated in the development of SCD pain episodes on the basis of clinical and pathologic observations [16]. Elevation of NGAL levels could be added to other biochemical markers of thrombogenesis. Tomer et al. [16], demonstrated elevated plasma fibrinogen, factor VII, fibrin-fibrogen complexes, fibrin(ogen) degradation products and D-dimer, coupled with decline plasma concentration of factor V and plasminogen.

NGAL represents an early, predictive, non-invasive biomarker that enforce the ability to institute potentially effective therapies for some clinical conditions in a timely manner [17,18] reported elevated NGAL levels in acute kidney injury earlier before detection of reduced kidney function.

The pathogenic role of NGAL for T-lymphocyte chemoattraction was demonstrated in patients with uveitis and has its role in the pathogenesis of uveitis [19]. This study for pathogenesis was also performed to identify putative molecular targets for therapeutic intervention.

Our study also showed a wide spread of NGAL values within each group (crisis, steady state and controls) with minor overlap between different groups. This spread in SCD is not unexpected because of the heterogeneity of disease severity among the patients. These changes in the range of NGAL levels and overlap was also reported by Sozzi et al. [20] in cancer patients.

Again, this variability may reflect, in part, the difficulty encountered to decide if a patient in crisis or steady state. We tried to avoid subjectivity in pain interpretation as far as possible. The samples categorized as (crisis) if the patient was admitted into hospital with acute pain. However, it is known that pain tolerance varies widely within any group of individuals. So, some of ‘steady state’ samples may have been obtained in the resolution phase of milder crisis that have been managed at home. Variation in the NGAL values may be related to the timing of samples collection of crisis group as it may vary from the first to the fifth day of hospital admission.

NGAL levels in steady state SCD is comparable to that in healthy controls. This reflects more of the significance of high NGAL levels observed in SCD with painful crisis. The clearance mechanisms of NGAL are not fully understood. Differential physiological clearance by the liver and kidney has not been fully explored and could represent another factor in the variability of NGAL levels in SCD patients [21,22].

An increase of NGAL was demonstrated in inflammatory bowel disease [4], adnocarcinomas as previously mentioned, specially of tissues exposed to high numbers of microorganisms. Recently it has been described that NGAL has a strong specificity for bacterial ferric siderophores and that NGAL acts as bacteriostatic agent by sequestering iron which is vital for bacterial growth [12]. The expression of the murine NGAL homologue, 24p3 is also up-regulated in response to inflammatory stimuli and in the uterus at the time of parturition where it is believed to be part of local inflammatory response associated with birth Liu et
al. [23]. D’Anna et al. [24]. Together with the data presented, this suggests that NGAL has its role as a part of the innate immune response to bacterial challenges, infection and inflammation. Neutrophils store NGAL in their specific granules represents the mobile source of NGAL [22,25]. Also the epithelial cells are important for local defence against infection showed strong induction of NGAL in response to inflammation [19,23].

This preliminary study has shown that acute painful crisis in SCD are associated with several folds increases of NGAL levels when compared with steady state. Although this work is exploratory in small sample size and requires further validation in more SCD populations, the findings suggest avenues for further studies.

With the increasing life span of patients with SCD, end-organ failure becomes an emerging problem [26]. We are encouraged by these results which suggest that monitoring of NGAL, perhaps in combination with other biomarkers (e.g. WBC, CRP and LDH), may be helpful during the episode of acute pain in SCD, determine the progress for development of acute chest syndrome and also predicting resolution of a crisis episode.

**References**

5. COWLAND J.B., SORENSEN O.E., SEHESTED M. and BORREGAARD N.: Neutrophil gelatinase associated lipocalin is up regulated in human epithelial cells by IL-1B but not by TNF-α. The Journal of Immunology, 171: 6630-6639, 2003.


