

## Reference Interval for Serum Neutrophil Gelatinase-Associated Lipocalin in Apparently Healthy Caribbean Population

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### ABSTRACT

**Aim:** Neutrophil gelatinase-associated lipocalin (NGAL) is emerging, as a promising biomarker for diagnosing acute kidney injury, yet its reference interval is not established in many populations. This report documents serum NGAL reference intervals in apparently healthy population in the Caribbean in relation to age, sex, body mass index (BMI) and blood pressure.

**Methods:** The blood pressure (BP), height and weight of 90 (37 males, 53 females) apparently healthy subjects aged between 17 and 83 years were measured. Fasting blood samples were collected and serum NGAL levels were measured with manual ELISA technique and 95% reference intervals for NGAL stratified by age and sex was established. The Statistical Package for the Social Sciences (SPSS) was used for statistical analysis.

**Results:** The results are expressed as mean  $\pm$  2 SD. The age, BP and BMI were similar in male and female subjects studied ( $p > 0.05$ ). NGAL levels showed a normal Gaussian frequency distribution and were similar in male and female subjects ( $p > 0.05$ ). The overall unisex reference interval for the population ( $n = 90$ ; age 17 – 83yrs.) was 73.3-85.6 ng/mL, while the gender reference intervals was 68.3-92.6 ng/mL for male and 73.2- 84.1 ng/mL for female and for age category  $< 60$ yrs ( $n = 82$ ; age 17 – 59yrs) was 73.3 – 84.1 ng/mL.

**Conclusion:** This study showed that apparently healthy male and female subjects with similar mean age, body mass index and blood pressure had similar

serum NGAL levels. The gender- and age-specific reference intervals determined in this study could therefore provide a suitable template for further studies in relation to establishing reference intervals for serum NGAL for this and other populations.

**Keywords:** *Biomarker, Caribbean, kidney disease, neutrophil gelatinase-associated lipocalin, noncommunicable diseases, reference interval.*

### INTRODUCTION

The Global Burden of Disease report shows high systolic blood pressure (SBP) was the leading risk factor, accounting for 10.4 million deaths, followed by smoking (7.10 million deaths), high fasting plasma glucose (6.53 million deaths), high body-mass index (4.72 million deaths), and short gestation for birth weight (1.43 million deaths).<sup>1</sup> It is well documented that the burden of noncommunicable diseases (CNCD's) is more devastating in low- and middle-income countries.<sup>1,2</sup> In Caribbean especially in Trinidad and Tobago, diabetes is one the five leading cause of mortality,<sup>3</sup> and also the major cause of end-stage kidney disease globally<sup>4,5,6</sup> with about 22-27% of diabetes patients visiting lifestyle disease clinics in the Caribbean having kidney disease.<sup>7</sup> More than 28% of the patients were on kidney replacement therapy (KRT).<sup>8</sup> Indeed, the prevalence of kidney disease as a complication from diabetes, hypertension or heart

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disease is increasing world-wide particularly in the developing countries<sup>9</sup> and diagnosing acute kidney injury (AKI) can be challenging because clinical signs might be asymptomatic or absent entirely. The population most susceptible to AKI are those aged >65 years, those with diabetes mellitus, chronic kidney disease (CKD), heart failure and anemia. Others include those who have been exposed to predisposing factors such as sepsis, major surgery or nephrotoxins. In addition, duration and severity of AKI is a well established risk factor for the development of complications such as a 10-fold increase risk of developing CKD and a 3-fold risk of developing end-stage kidney failure.<sup>10-12</sup> The identification of early kidney injury is therefore very important, especially in the light of the recently identified biomarker, neutrophil gelatinase-associated lipocalin (NGAL) secreted by the kidney during acute injury, which has been shown to have biochemical capacity to detect early injury to the kidney and many studies have identified its potential use in the diagnosis and management of kidney injury.<sup>13-18</sup> Neutrophil gelatinase associated lipocalin (NGAL) belongs to the super family of the lipocalin. The family generally shares common molecular organizations that are composed of eight  $\beta$ -strands arranged in a complex  $\beta$ -barrel structure delineating a calyx shape, which also represents their binding site.<sup>15,16</sup> NGAL is highly expressed in the tubular epithelium of the distal nephrons of the kidney and is released from tubular epithelial cells following damage such as that happens in acute kidney injury to regulate the release of catalytic iron from injured kidney cells. The NGAL protein exists in three distinct molecular forms; the 25 kDa monomer, the 45 kDa homodimer generated by dimerization of the two identical NGAL monomers and the larger 135 kDa heterodimer generated by association of the monomer with the 92 kDa MM-9, also called gelatinase B.<sup>19,20</sup> NGAL expression rises about two-fold in humans and rodents in response to renal tubular injury and appears so rapidly in urine and serum that it is useful as an early biomarker of kidney disease.<sup>21</sup>

Neutrophil gelatinase-associated lipocalin has been identified to have the clinical potential to detect early injury to the kidney and its reference intervals in urine and blood have been established in other population but not in the Caribbean population. In an attempt to enhance the clinical application of

this important biomarker in the diagnosis and management of kidney injury, this study aimed to determine the reference interval for serum NGAL in apparently healthy adult subjects of Caribbean origin.

## MATERIALS AND METHODS

**Patient's recruitment:** 90 adult subjects (53 females, 37 males), aged between 17 and 83 years and residing in Trinidad were recruited for the study. Recruitment of subjects was from a pool of workers at the Water and Sewage Authority (WASA), Mount Hope, Trinidad, staffs and students from the University of the West Indies (UWI), St Augustine Campus, Trinidad as well as staffs of Eric Williams Medical Science Complex (EWMSC), Mount Hope, Trinidad. The subjects were recruited by advertisement and announcement. All subjects gave informed voluntary consent to participate in the study after the researcher has explained the study protocol and the benefits of participating in the study.

**Eligibility criteria:** All the subjects had fasting plasma glucose < 7.0 mmol/L<sup>22</sup> or blood pressure < 135/90 mmHg<sup>23</sup> or an estimated glomerular filtration rate >90 mLs/min/1.73m<sup>2</sup> calculated using the CKD-EPI equation.<sup>24</sup> Subjects diagnosed with conditions such as kidney disease, diabetes, hypertension, heart disease, cancer, infectious or inflammatory conditions or taking medications for such or those who declined voluntary consent were excluded from participating in the study.

**Study protocol:** Five (5) millimeter of blood was collected from each of the subjects after an overnight fast (10 – 12 hr) into red-top tube without anticoagulant for serum NGAL measurement. The serum were separated and stored frozen at -80°C until laboratory analysis. Anthropometric indices such as weight (measured in kg with clinic measuring scale), height (measured in meters with clinic measuring ruler) and clinical information (age, gender, education, occupation, ethnic group, any medical history including medications) were obtained. The subjects' blood pressures were measured on the dominant arm in a sitting position after resting for about ten minutes on the study day. The Ethics Committees of the University of the West Indies reviewed and approved the study protocol.

**Laboratory analysis**

Serum NGAL was analysed manually using ELISA kit from Biovendor Laborotoni medicina a.s. Karasek 176/1 621 00, Brno, Czech Republic. The ELISA tests were performed in duplicates and the calculated intra- and inter-coefficient of variations were 7.8% and 9.5% respectively.

**Statistics and calculations**

All data were entered into Excel and the statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Inc., 233 South Wacker Drive, Chicago, USA). Differences in quantitative variables such as blood pressure (BP), height, weight, BMI (calculated as weight (kg)/height (m<sup>2</sup>) and serum NGAL levels for the male and female gender categories were determined using t-tests while chi-squared ( $\chi^2$ ) test was used for categorical variables that included educational status, employment status and social habits. The reference values were calculated from the 95% confidence intervals.

**RESULT**

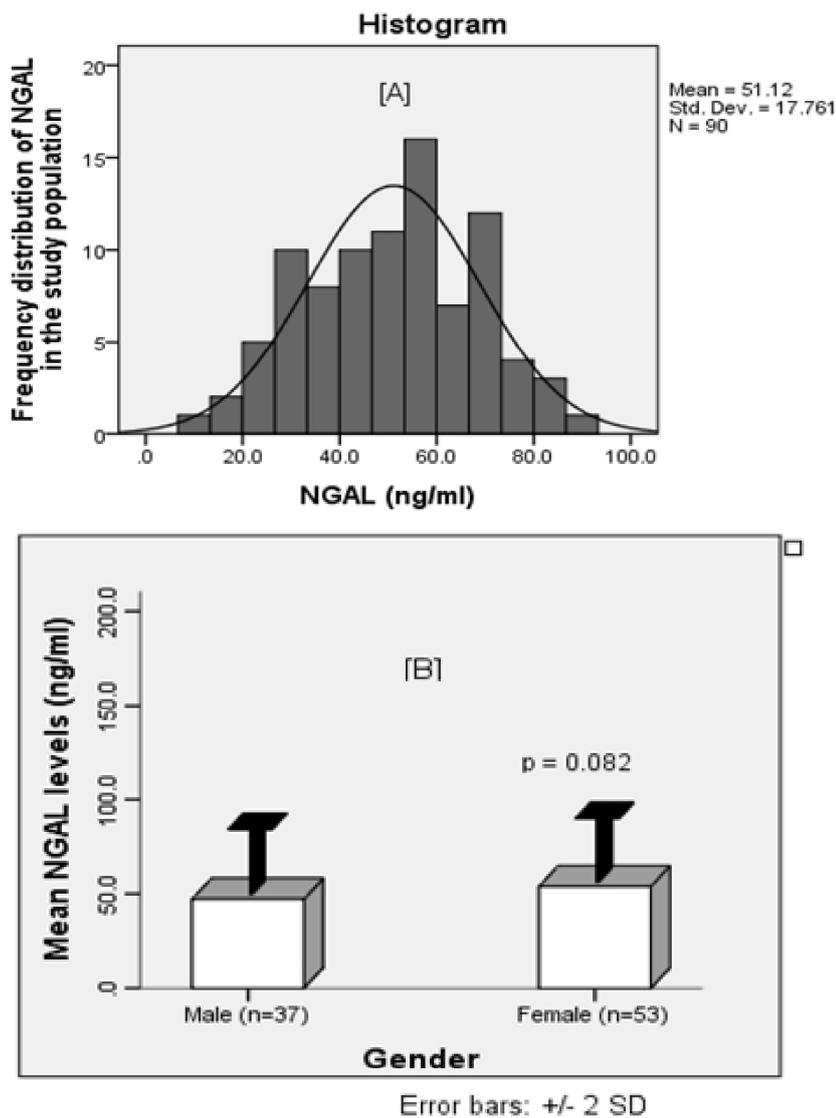
The results are expressed as mean ( $\pm$ SD) and in percentages (%) as appropriate. Table 1 shows the demographic characteristics of the 90 (53 females, 37 males) apparently healthy subjects studied. The subjects were drawn from different ethnic background of African, East Indian and mixed ethnic origin. The majorities of the subjects had higher education and were employed while 45% admitted to the use of alcoholic beverages. Table 1 also shows that age, blood pressure (BP), BMI and serum NGAL levels were similar in male and female subjects studied ( $p > 0.05$ ). Fig. 1 shows that NGAL had a normal Gaussian frequency distribution and were similar in male and female subjects ( $p > 0.05$ ). Table 3 shows the 95<sup>th</sup> percentile interval of NGAL for the sample population, in gender and in <60 years age category. The gender reference intervals for serum NGAL are 68.3 – 92.6 ng/mL for male and 73.2 – 84.1 ng/mL for female while the age-specific reference interval for subjects <60 years old ( $n = 82$ ; age 17 – 59yrs.) was 73.3 – 84.1 ng/mL. The overall reference interval for the population ( $n = 90$ ; age 17 – 83yrs.) was 73.3 – 85.6 ng/mL

**Table 1:** Gender-related characteristics of the study population

Parameter	All subjects (n= 90)	Males (n = 37)	Females (n= 53)
African descent, n, (%)	42 (46.7)	18 (48.6)	24 (45.3)
East Indian descent, n, (%)	34 (37.8)	12 (32.4)	22 (41.5)
Mixed ethnicity, n, (%)	14 (15.6)	7 (18.9)	7 (13.2)
Employed, n, (%)	76 (84.4)	32 (86.5)	44 (83.0)
Unemployed, n, (%)	14 (15.6)	5 (13.5)	9 (17.0)
Alcohol drinkers, n, (%)	41 (45.6)	22 (59.5)	19 (35.8)
Cigarette smokers, n, (%)	9 (10.0)	5 (13.5)	4 (7.5)
Primary school, n, (%)	9 (10.0)	5 (13.5)	4 (7.5)
Secondary school, n, (%)	38 (42.2)	18 (48.6)	20 (37.7)
Tertiary education, n, (%)	43 (47.8)	14 (37.8)	29 (54.7)
Age (yr.)	41.6 $\pm$ 13.3	44.1 $\pm$ 13.7	39.8 $\pm$ 12.9
Body mass index, (kg/m <sup>2</sup> )	27.0 $\pm$ 5.8	26.8 $\pm$ 5.3	27.2 $\pm$ 6.2
Systolic BP (mmHg)	122.3 $\pm$ 16.0	122.6 $\pm$ 12.9	122.1 $\pm$ 17.9
Diastolic BP (mmHg)	77.7 $\pm$ 11.6	76.7 $\pm$ 11.2	78.4 $\pm$ 11.9

**Table 2:** Gender- and age-specific Neutrophil Gelatinase-associated Lipocalin 95<sup>th</sup> percentile reference intervals

Parameter, Mean ± SD	All subjects (n= 90)	Males (n = 37)	Females (n= 53)	<60yr. subjects (n = 82)
NGAL (ng/mL)	79.4 ± 6.1	80.5 ± 12.2	78.6 ± 5.4	78.7 ± 5.4
Reference intervals	73.3 - 85.6	68.3 - 92.6	73.2 - 84.1	73.3 - 84.1



**Fig. 1:** Shows that NGAL had a normal Gaussian frequency distribution [A] and were similar in male and female subjects [B]

## DISCUSSION

The results of this study showed that, serum NGAL levels had a normal frequency distribution with similar levels in male and female subjects and in the <60years age category. While the overall unisex reference interval for the population (n = 90; age 17 – 83yrs.) was 73.3 - 85.6 ng/mL, the reference interval for the < 60years. (n = 82; age 17 – 59yrs.) was 73.3 – 84.1 ng/mL. The gender reference interval was 68.3 - 92.6 ng/mL for male and 73.2 - 84.1 ng/mL for female respectively.

The finding of similar levels of NGAL in male and female subjects in this study could be explained by the similarity in the age, anthropometric indices and blood pressure of the subjects recruited for the study (Table 1). This similarity in age, anthropometric indices and blood pressure perhaps makes the study subjects the most suitable group for assessing the levels of NGAL in a healthy population especially for establishing reference intervals. Indeed, the importance of establishing reference values for NGAL based on age and gender in healthy populations has been recommended by authors of previous studies on NGAL.<sup>25</sup>In line with this recommendation, the present study showed that apparently healthy male and female subjects with similar mean age, BMI or blood pressure had similar serum NGAL levels. NGAL is highly expressed in the tubular epithelium of the distal nephrons of the kidney and is released from tubular epithelial cells following damage such as that happens in acute kidney injury to regulate the release of catalytic iron from injured kidney cells.<sup>16,20,26-30</sup>After injury to the kidney, NGAL mRNA protein expression is rapidly induced in proximal tubular epithelial cells of the kidney.<sup>16,20,26-30</sup>In this light, several studies, have therefore demonstrated the potential of NGAL as early biochemical marker for diagnosing acute kidney injury, although the establishment of reference interval for NGAL is still at research stage and needs further investigations.<sup>15,18,31 - 36</sup>Thus, the current finding of similar reference range for males and females in this study is in contrast with a previous report in urine and plasma of NGAL values. Makris K et al determined plasma NGAL reference interval in 200 plasma and urine samples (137 males, 63 females) from healthy subjects using automated biochemical analyzer (Abbot-Architect-8000) and reported higher reference intervals in males (38.7 - 157.6 ng/mL) than females (24.4 - 142.5 ng/mL).<sup>33</sup>Again, while this

other study that measured NGAL in urine or plasma samples used automated biochemical analyzers, NGAL measurement in this study was performed using ELISA manual method (Bio vendor Laborotonimedicinaa.s. Karasek 176/1 621 00, Brno, Czech Republic). This limitation therefore made comparison of the proposed reference intervals challenging for different studies and populations. Nevertheless, the comparatively lower levels of serum NGAL observed in the apparently healthy subjects in the present study were a confirmation that the study subjects were indeed free from acute kidney injury.

The gender- and age-specific reference intervals determined in this study could therefore provide a suitable template for further studies in relation to establishing reference intervals for serum NGAL for this and other populations. The reports of previous studies on reference intervals for urine and plasma NGAL have been contentious. For example, a study on 174 urine samples from healthy subjects measured with automated biochemical analyzer (Abbot Architect) showed that, normalized urinary NGAL to creatinine had a significant gender- and age-related differences and the authors suggested normalization of urinary NGAL to creatinine before clinical application of the reference interval.<sup>34</sup>The recommendation for the normalization of urinary NGAL to creatinine is contentious given the need for further investigations in different population groups before its application as urine NGAL reference interval.<sup>36</sup>

The current proposal of upper reference interval of <54ng/mL and <71.6ng/mL for individuals <60years and >60years old respectively indicates higher reference range for older persons compared to their younger counterparts.<sup>37</sup>Similarly, a previous study that measured serum NGAL levels in 454 healthy donors using automated biochemical analyzer (Cobas 6000 series c501module) proposed age specific reference intervals of <116.52 µg/L in the 21 - 44 year age group and <126.9 µg/L in the 45 - 75 year age group<sup>34</sup>. Thus, it would seem that different studies have proposed different reference intervals for gender and different age categories<sup>32-34,36</sup>. Stejskal *et al.*, measured NGAL with an ELISA method in serum samples of adult, non-obese subjects. The NGAL values observed in the 53 men (mean: 86.3 ig/L; SD: 43.0 ig/L; median: 78.8 ig/L) were not significantly different from those found in the 83

women (mean: 88.9  $\mu\text{g/L}$ ; SD: 38.2.0  $\mu\text{g/L}$ ; median: 80.0  $\mu\text{g/L}$ ). This finding was in accordance with the findings in the present study.<sup>38</sup>

Although, we do recognize that the sample size of the healthy subjects used was relatively small compared with the other studies reported, it has however provided a valuable template for further studies on reference intervals in this and other populations.<sup>32-34,36</sup> Nonetheless, it is worth noting that even well-designed, large reference interval studies may not satisfy all the pre-requisite conditions for setting reference intervals due to some subjective factors in the design and interpretation, such as the choice of population, the numbers included, the statistical techniques used, and the method of outlier exclusion which require professional judgment and may be done differently in different locations, even when the same data set is being considered. Nevertheless, this is recognized as one of the limitations of the study that might have emanated after the subjects were stratified based on the gender and age group. Overall, the total number of the subjects recruited was one hundred and twenty which might have the statistical power, but this apparently might have disappeared particularly after the outliers removal and stratification into age and sex categories. Again, while the other studies that measured NGAL in urine or plasma samples used automated biochemical analyzers, NGAL measurement in this study was performed using ELISA manual method (Bio vendor Laborotoni medicina a.s. Karasek 176/1 621 00, Brno, Czech Republic)<sup>32-34,36</sup>. These limitation made comparison of the proposed reference intervals challenging for different studies and populations.

### CONCLUSION

The reference intervals established for NGAL in the <60years age category and gender is the first in the Caribbean and could therefore serve as a template for further investigations on the reference intervals for NGAL in this and other populations.

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