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RECEIVER OPERATING CURVE (ROC) ANALYSIS FOR FLUOROSIS USING SIMPLE BLOOD PARAMETER NEUTROPHIL LYMPHOCYTE RATIO

S. Gupta[1], A. N. Poddar[2*], C. Kumar[3] and S. Pervez[4]

[1,2]School of Studies in Life Sciences, [4]School of Studies in Chemistry, Pt. Ravishankar Shukla University, Raipur- 492010, India.
[3]Govt. RRM PG College, Surajpur-497229, Chhattisgarh, India.
*e-mail:adinpod@gmail.com

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ABSTRACT : The study aims to assess the preclinical stage of fluorosis i.e. Non skeletal fluorosis in human populations using a simple blood parameter, Neutrophil Lymphocyte Ratio as a predictive tool and includes different fluorosis categories (Dental, Skeletal & Non skeletal) surveyed out in the endemic villages and compared with the control population of non endemic area. Study Populations belonging to endemic villages Kowataal, Fulsar, Mahuapaani, Korbi and Amatikra within the block Podi uproda, distt. Korba situated in Chhattisgarh state of central India were cross sectionally studied. Segregation was done on the basis of Suspect (non-symptomatic) and Confirmed (Dental and Skeletal) cases of fluorosis and neutrophil to lymphocyte ratio calculated. A total 180 individuals were included in the study, out of which 67.33 % were confirmed cases of fluorosis. A significant decline in neutrophil to lymphocyte ratio was observed upon chronic fluoride ingestion. In the Receiver Operating Characteristic curve analysis of obtained NL ratios, the overall cut off value for fluorosis was obtained as ≤ 2.379 in the selected endemic region irrespective of symptoms of fluorosis *i.e.* dental or skeletal. The results concluding that The NL ratio can be used as a simple parameter for preclinical identification of fluorosis in fluoride exposed populations. However, urine and blood fluoride analyses of the subjects are also needed for further confirmation.

Key words : Neutrophil lymphocyte ratio, non skeletal fluorosis, ROC, skeletal fluorosis.

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INTRODUCTION

Preceding dental and skeletal fluorosis, some preclinical changes take place in the blood and body due to acute or chronic ingestion of fluoridated drinking water and have been considered under non-skeletal manifestations. Such manifestations are often overlooked due to the misconception that fluoride affects only bones and teeth (Raghuvansi *et al*, 2010). Fluoride has multiple effects on human health. A few are characterized by mineralization changes in the calcified tissues resulting in dental fluorosis and skeletal deformities. Besides other metabolic effects, visceral organs like liver and kidneys are also susceptible to toxic effects of fluoride and pathological changes in these vital organs can occur even before overt clinical signs of F intoxication (Shashi, 2002). Fluoride exposure is also associated with oxidative damage to RBCs, liver and kidney tissues. Consequent to oral exposure, fluoride is rapidly absorbed to reach systemic circulation. In blood, about 75% of it remains free in plasma and about 5% remains bound to plasma proteins. The rest of the blood F is found mainly inside RBCs or with their membrane (Swarup and Dwivedi, 2002). Neurological manifestations, like headache, insomnia (lack of sleep), lethargy (fatigue), depression, polyuria and polydipsia have been reported in populations with endemic fluoride (Sharma *et al*, 2009). Besides, skeletal and dental fluorosis, excessive consumption of fluoride may lead to muscle fiber degeneration, low hemoglobin levels, excessive thirst, headache, skin rashes, nervousness, depression, etc (Meenakshi, 2006). Pre skeletal stage of fluorosis is associated with occasional complains of pains in small points of limits and back, which simulate rheumatoid arthritis and ankylosing spondylosis (Krishna and Kiran, 2013).

In earlier studies (Zahorec *et al*, 2008; Zazula *et al*, 2008; Papa *et al*, 2008; Halazun *et al*, 2008; De Jager *et al*, 2010; Kaol *et al*, 2010; Indavarapu and Akinapelli, 2011; Tomita *et al*, 2011; Proctor *et al*, 2012; Kim and

Choi, 2012; Selcuk *et al*, 2012; Absenger *et al,* 2013; Balta *et al*, 2013; Szkandera *et al*, 2013; Cakýcý *et al*, 2014; Halazun *et al*, 2014 and Duzlu *et al*, 2015). The ratio between neutrophils and lymphocytes (N/L) count has been investigated as a new predictor for various diseases and risk, along with Receiver Operating Characteristic (ROC) curves constructed to evaluate the sensitivity and specificity of the study and to obtain optimal cut off values. Although, fluoride has also been found to alter mitotic activity of lymphocytes and influence the function of neutrophils (Pacauskiene *et al*, 2005) no work has yet been done on the neutrophil to lymphocyte ratio in fluorosis individuals. This work deals with studies on the neutrophil to lymphocyte ratio in fluorosis subjects as compared from control subjects and Receiver Operating Curve analysis of obtained NL ratios.

The district Korba is the power hub of Chhatisgarh state.The problem of fluoride in drinking water has been recently discovered in the study area, Block Podi uproda by Public Health Engineering Department (PHED). In a previous work, a survey in human population belonging to ten fluoride endemic areas was conducted for dental and skeletal symptoms (Gupta *et al*, 2015). Since, dental and skeletal changes are considered to be irreversible advanced stages; hence the present study was aimed to investigate fluorosis within the population in its preclinical stage.

MATERIALS AND METHODS

With a view to survey out the prevalence and type of fluorosis, an epidemiological cross-sectional field study was conducted during the year 2014- 2015, amongst inhabitant populations residing in the endemic and non-endemic villages, with the help of a pre-formed questionnaire and a consent form were also signed by the individuals explaining the purpose of the study. The study included, Kowataal, Mahuapani, Fulsar, Amatikra, Korbi, as the fluoride endemic villages located in the study area (block Podi uproda, district Korba) in comparison with the non-endemic area. Sampling was done with the help of simple random sampling method. Drinking water samples from the study areas were collected and estimated for the fluoride content with the help of Thermo scientific orion 9609 BNWP ion selective fluoride electrode. Procedures followed were approved by the Institutional Human Ethics Committee, Pt. Ravishankar Shukla University, Raipur, CG. Neonates, children, pregnant women and patients with other severe & chronic diseases were excluded from the study. Since no previous data were available in the literature, the prevalence of disease was calculated by a pilot study.

Study design : The sample size calculation for cross sectional field study was done by Epi Info Statistical Software 7 version (Stat Calc) www.cdc.gov.in. In all 180 individuals (30 individuals from each endemic village and 30 from control area) were included in the study. Subject selection was done from fluoride endemic and non endemic zones of district Korba.

Sample collection : 2-3mL blood was drawn from the individuals by antecubital vein puncture via disposable syringe and kept in heparinized vials. The blood samples were transported at 4°C to the laboratory for further analyses. Complete blood picture (CBP) was obtained through the Semi auto analyzer (Model CHEM 400), Electronics India. The neutrophil to lymphocyte ratio (N/L Ratio) was determined from the CBC reports obtained.

Statistical analysis : ANOVA analyses were done by SPSS software (version 16.0) and the ROC (Receiver Operating Characteristics) curve analysis was done in XLSTAT software.

RESULTS

Prevalence : The mean drinking water fluoride level (mgL^{-1}) and the prevalence of dental and skeletal fluorosis in five fluoride endemic village of the study area block-Podi uproda is shown in Table 2.

Total WBC count, Neutrophil and Lymphocyte count & ratio (NLR) is depicted in Table 3 in control and fluorosis subjects from individual endemic villages.

Neutrophil to Lymphocyte ratio's: Human population categorized as dental, skeletal (confirmed) and non skeletal (suspected) cases of fluorosis (Plate 1).

Table 1 : Subject selection criteria amongst human population of endemic and non endemic villages.

Village	Population	No. of individuals	Details of participants
Non endemic (n=01)	Control	30	No exposure to fluoride in drinking water, non diseased.
Endemic (n=5)	Suspect	50	Exposed individuals from endemic villages with no visible symptoms of disease (i.e. 10 individuals from each village).
	Confirmed	100	Individuals with visible symptoms of disease either dental or skeletal (i.e. 10 dental and 10 skeletal cases from each village).

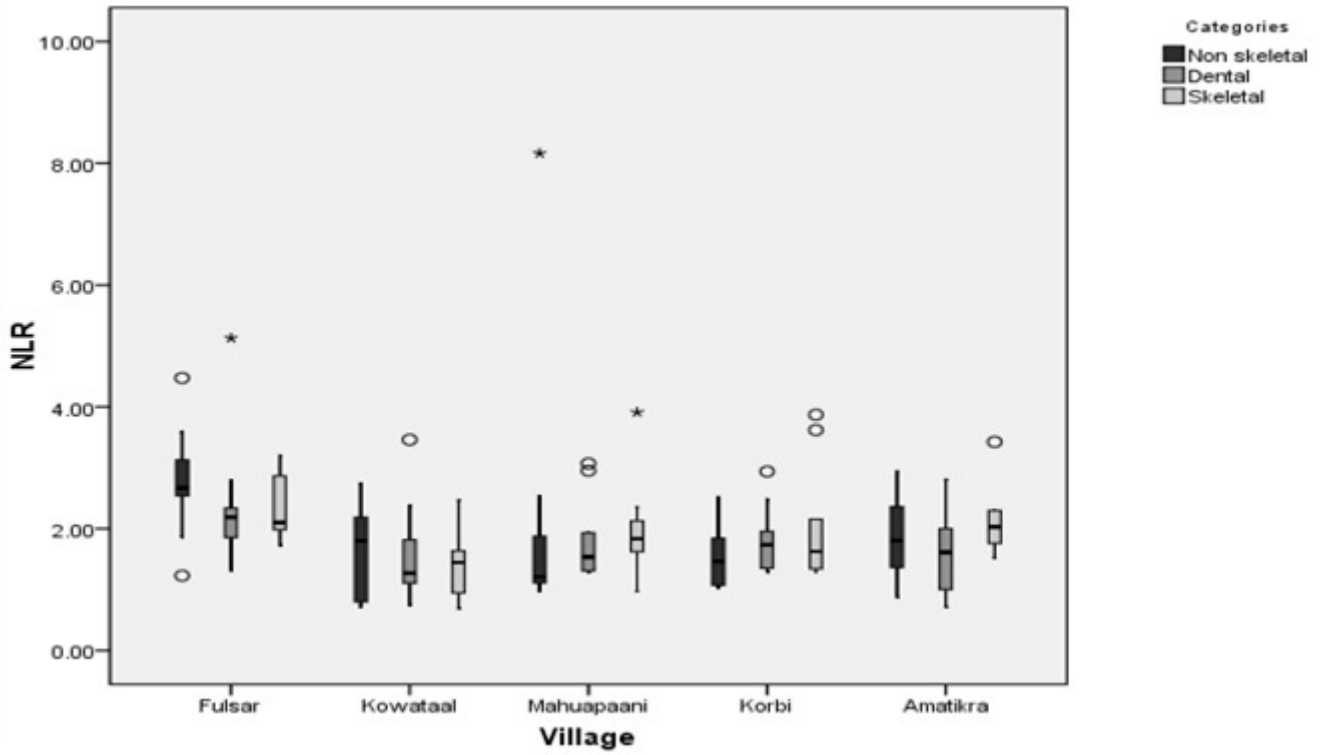

Plate 1 : Neutrophil to lymphocyte ratio in different fluorosis categories in five fluoride endemic villages.

Table 2 : Prevalence of fluorosis (n=300).

Village	F(mg L^{-1})	Skeletal	Dental
Fulsar	5.5	0.77	0.87
Kowataal	1.16	0.43	0.67
Mahuapaani	4.42	0.50	0.73
Korbi	3.43	0.83	0.83
Amatikra	7.56	0.75	0.83

Table 3 : Hematological parameters and NL Ratio in study population.

Village Mean fluoride	Control (0.75±0.19)	Fulsar (5.5±1.85)	Kowataal (1.16±1.15)	Mahuapaani (4.42±0.80)	Korbi (3.43±1.08)	Amatikra (7.56±2.35)	P value
WBC	7.34±2.35	9.90±3.54	6.99±1.59	5.91±1.27	7.53±1.95	6.73±2.36	<0.001
NEUT	69.81±4.22	55.34±10.01	48.64±9.97	51.47±10.93	58.14±7.99	54.00±11.32	<0.001
LYMPH	26.94±6.48	23.91±4.62	34.40±8.28	30.59±7.68	35.26±7.59	29.99±8.57	<0.001
NLR	2.73±0.12	2.47±0.15	1.56±0.12	1.94±1.24	1.79±0.13	1.85±0.12	<0.001

*Mean ± SD (n=180 for blood samples); in each line alphabets in subscripts represents significance (P< 0.001) within the villages.

ROC aanlysis : To predict fluorosis in human populations in its pre-clinical stages, A cut off value for non skeletal fluorosis for NL ratio was obtained for overall and individual villages using ROC curve analysis, ROC curve is depicted with AUC (area under curve), PV (predictive values), LV(Likelihood ratio's) along with sensitivity and specificity of the computed data.

DISCUSSION

The fluoride content in potable water of the study area (Table 2) was observed to range between more than 1.16 and 7.56 ppm. The prevalence of fluorosis in the study population (Table 2) was observed to be between 0.43-0.83(skeletal fluorosis) and 0.67-0.83(dental fluorosis). Since, the socio economic status of the study populations, in both control and endemic cases were same and below poverty, the normal ranges of the hematological parameters were observed to be on the lower side (Table 3) due to poor nourishment, dietary habits and lack of awareness. Deficiency of adequate health supplements further augmented the deleterious effects of fluoride in the form of dental and skeletal deformities. However, individuals lacking visible deformities may be considered most susceptible and may be speculated to develop the same after a short duration of exposure based on their internal tolerance to the toxicant. In case of chronic fluoride toxicity a significant decline (P<0.001) in N/L ratio was observed in fluoride endemic populations compared from control (2.73±0.12) along with conditions of neutropenia. However, an augmentation in the

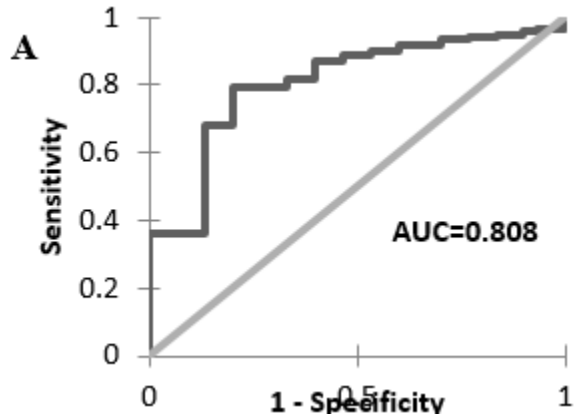

Fig. A : The cut off value for fluorosis (Non skeletal) in overall five endemic villages. If the N/L ratio d 2.379 with Sensitivity of 0.793 & Specificity of 0.800, +PV: 0.799, — PV: 0.795, +LR: 0.799, -LR:0.795 in ROC analysis (AUC = 0.808, P value <0.001).

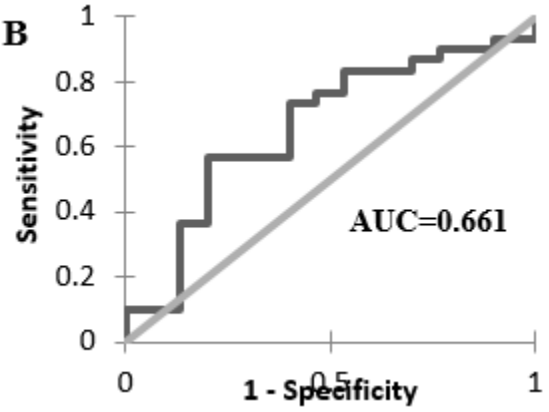

Fig. B : The cut off value for fluorosis (Non skeletal) in population of Fulsar village. If the N/L ratio ≤ 2.350 with Sensitivity of 0.567 Specificity of 0.800, +PV: 0.739, -PV: 0.649, +LR: 2.833, -LR: 0.542 in ROC analysis (AUC = 0.661, P value <0.001).

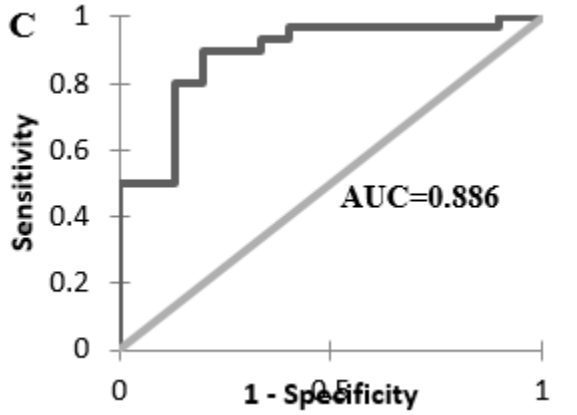

Fig. C : The cut off value for fluorosis (Non skeletal) in population of Kowataal village. If the N/L ratio ≤ 2.379 with Sensitivity of 0.900, Specificity of 0.800, +PV: 0.818, -PV: 0.889, +LR: 4.500, -LR: 0.125 in ROC analysis (AUC = 0.886, P value <0.001).

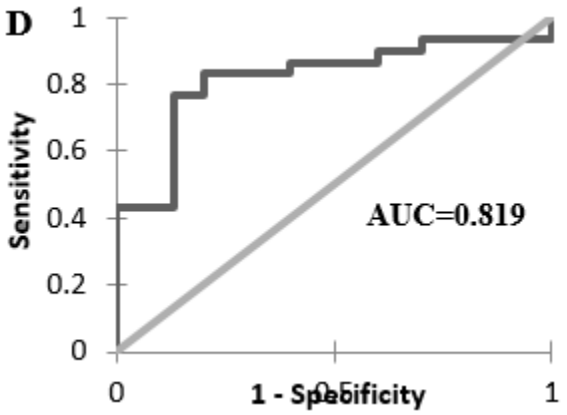

Fig. D : The cut off value for fluorosis (Non skeletal) population of Mahuapaani village. If the N/L ratio ≤ 2.350 with Sensitivity of 0.833 Specificity of 0.800, +PV: 0.806, -PV: 0.828, +LR: 4.167, -LR: 0.208 in ROC analysis (AUC = 0.832, P value <0.001).

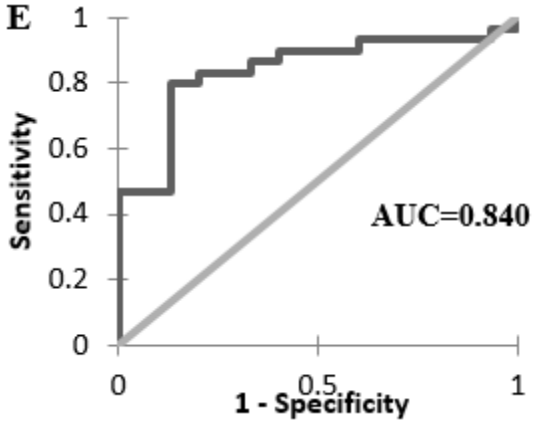

Fig. E : The cut off value for fluorosis (Non skeletal) in population of Korbi village. If the N/L ratio d" 2.050 with Sensitivity of 0.800 Specificity of 0.867, +PV: 0.857, -PV: 0.813, +LR: 6.00, -LR: 0.231 in ROC analysis (AUC = 0.840, P value <0.001).

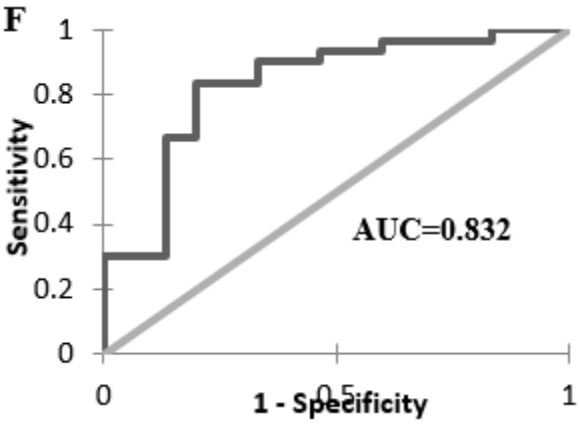

Fig. F : The cut off value for fluorosis (Non skeletal) in population of Amatikra village. If the N/L ratio ≤ 2.363 with Sensitivity of 0.833 Specificity of 0.800, +PV: 0.806, -PV: 0.828, +LR: 4.167, -LR: 0.208 in ROC analysis (AUC = 0.832, P value <0.001).

Fig. A-F : Receiver Operating Characteristics (ROC) Curve in N/L Ratio in overall (A) five villages and individual endemic villages- Fulsar (B), Kowataal(C), Mahuapaani (D), Korbi (E) and, Amatikra (F), respectively.

lymphocytes was also seen. In significant differences in N/L ratio existed among non skeletal, dental and skeletal categories (P=0.696) and among categories in different villages (P=0.812) indicating that the changes in the blood due to fluoride are irrespective of the fluorosis type (Plate 1).

Results of NL ratio of thirty samples from each village with diverse fluorosis categories (Total=150) show significant differences between the villages (P<0.05), but insignificant differences existed between the various fluorosis categories (P=0.696) and between their interactions (P=0.812) (Plate 1).

ROC analysis in case of fluorosis was also done in the present study to predict the cut off value for the disease fluorosis, irrespective of the fluorosis categories and symptomatic identification of the cases. The overall cut off value for N/L ratio in the five endemic areas (Fig. 1A–F) was found to be 2.379 with sensitivity of 0.793, specificity of 0.800 (P < 0.001), whereas, cut off value for individual villages viz., Fulsar (2.350), Kowataal (2.379), Mahuapaani (2.350), Korbi (2.050) and Amatikra (2.363) were also found significant (P<0.001).The area under curve (AUC) is an important determinant of good prediction in case of ROC analysis if in the range of 0.70-1.0. Similar result is found for AUC in the present predictive study for NL ratio and fluorosis.

CONCLUSION

Besides high concentrations of fluoride in potable water, poor socio economic status and nutritional deficiency also contribute to fluorosis in exposed individuals from endemic regions. The NL ratio can be used as a simple parameter for preclinical identification of fluorosis in fluoride exposed populations.The cut off value obtained for different villages may help in the prediction *i.e.*, if any individuals residing in an endemic area and consuming the same high fluoride containing drinking water but no visible symptoms of dental or skeletal fluorosis is there but the NL ratio is less then equal to the ROC curve cut off value computed for that population then individual may be considered in a preclinical stage of fluorosis and may develop symptoms of fluorosis in subsequent years. However, urine and blood fluoride analyses of the subjects are also needed for further confirmation.

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Conflict of interest

The authors have no conflict of interest in this study.

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