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RESEARCH ARTICLE

CORRELATIONAL STUDY OF NEUTROPHIL-TO LYMPHOCYTE RATIO AND BIOMARKERS (IL-6, CRP, ESR, FERRITIN) WITH COVID-19 SEVERITY

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Abstract

Background: India is 5th worst hit nation by COVID-19 with 5.2 lacs deaths attributable to the disease. To counteract this global tragedy, many research articles have highlighted the role of various haematological and inflammatory biomarkers in assessing the severity of the disease.

Materials and Methods: A retrospective analysis of 100 RT-PCR confirmed COVID-19 patients was undertaken. The patients were initially categorised into mild, moderate, and severe categories based on SpO2 levels & clinical symptoms at the time of admission. The blood sample was analysed for haematological and inflammatory biomarkers including NLR, IL-6, CRP, ESR and Serum ferritin. The data obtained was compared between severe and non-severe (mild+moderate) categories.

Results: On correlating NLR with disease severity, patients with non-severe disease had a mean of 3.7 whereas it was 6.7 in the severe group, signaling that patient with severe disease had significantly high NLR compared to non-severe patients. The inflammatory markers studied, ESR, CRP & Serum ferritin- all three of them showed markedly increased mean values in severe patients. However, in our study, IL-6 levels were not found to correlate with severity of COVID-19. On performing ANOVA 't' test, NLR, ESR and serum ferritin were found to be significantly correlated with disease severity (p-value<0.05) but no significant correlation was found with IL-6.

Conclusions: Our study highlights the importance of taking into consideration the values of NLR, ESR, CRP and serum ferritin as a screening tool at the time of initial presentation of COVID-19 patients

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to triage those requiring intensive care for optimum utilisation of limited resources.

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Introduction:-

The corona virus disease (COVID-19) was declared pandemic by WHO on 11th March 2020. First COVID-19 case in India was reported from Kerela (January 2020).[1] In India, till September 2022, there have been 44,539,046 confirmed cases of covid-19 with 528,355 deaths.[2]

Many research articles/meta-analysis have been published highlighting severely affected patients in which dysregulation of immune response results in development of cytokine storm with hyperinflammation resulting in increased mortality.

In the present study, we analyzed the correlation between NLR and other biomarkers like ESR, CRP, Serum ferritin & IL-6 with clinical severity of COVID-19 infection. The evaluation of results obtained may aid the treating physicians to comprehend in a better way, as to how these biomarkers can predict the severity of COVID-19 infection and triage those requiring close monitoring and intervention thereby lowering the mortality rate of this disease.

Material and Methods:-

It is a retrospective study in which the medical records of hundred RT-PCR confirmed COVID-19 patients who got admitted at Sharda Hospital during first wave of COVID-19 i.e., September 2020 to October 2020 were screened. At the time of admission, the patients were categorised into mild, moderate, and severe category as per ICMR COVID guidelines by Ministry of Health and Family Welfare (MOHFW), Government of India revised on 14/01/2022which takes into consideration SpO2 levels and respiratory rate. Patients without shortness of breath or hypoxia were categorized as mild, those with respiratory rate ≥24/min and SpO2 levels between 90% to ≤93% on room air were categorized as moderate, while those with respiratory rate >30/min and SpO2 levels <90% on room air were categorized as severe category. The initial blood sample taken at the time of admission was analysed for haematological and inflammatory biomarkers including NLR, IL-6, CRP, ESR and Serum ferritin.

All COVID positive blood samples received in the hematology laboratory of our hospital were processed by Sysmex XN-1000 analyzer, and automated data thus received was used to determine the differential count of white blood cells and calculate NLR. ESR was estimated using the Westergren tube method. Ferritin was estimated in the biochemistry laboratory by automated immunoassay using system VITROS 5600. IL-6 was estimated in the biochemistry laboratory by ELISA using LISA PLUS MICROPLATE READER. CRP was evaluated in the microbiology laboratory by latex agglutination method.

The laboratory parameters thus obtained were compiled and analyzed as per the category or severity of COVID-19. A comparative evaluation of all the parameters was done between mild, moderate, and severe category.

However, for ease of comparing our data with other studies done in COVID patients, mild and moderate categories were clubbed together into non-severe category and data was compared with severe category.

Those patients who were >60 and <1year of age, pregnant females, patients having community acquired pneumonia, ischemic heart disease, acute pancreatitis and on immune-suppressive drugs were excluded from study.

Statistical Analysis: -

All the data analyzed was entered into a word excel sheet. The continuous or quantitative data analysis is presented as mean +/- SD (min-max) and the results of categorical or qualitative data has been processed in percentages (%) and proportions. SPSS software version 26 was used for comparison and descriptive statistics. Comparison between qualitative data was analyzed by chi-square test. Comparison of quantitative variables between the group was analyzed by ANOVA ‘t’ test according to distribution. A p value of ≤ 0.05 was considered significant.

Results:-

This study was limited to patients in between age group of 1-60 years. A total of 100 patients were reviewed of which 68% were placed in non-severe category i.e., mild and moderate COVID based on their SpO2 levels, while 32% were placed in severe category (Table 1).

Table 1:- Master chart depicting demographic characteristics and biomarkers with severity of COVID-19 infection.

			NORMAL RANGE	NON-SEVERE (mild + moderate) (n=68)	SEVERE (n=32)	p-value
				Mean(range)	**Mean(range)**	
DEMOGRAPHIC CHARACTERISTICS	**Age**		-	41.01 (10-59) Median = 43	43.9 (13-59) Median = 45	-
	Sex	**Male**	-	52 (76.4%)	28 (87.5%)	-
		Female	-	16 (23.5%)	4 (12.5 %)	-
LABORATORYPARAMETERS	**NLR**		<3.5	3.72 (0.5-23.2)	6.73 (0.78-23.7)	0.016
	ESR		0-55 mm/hr	24.25 (4-102)	45.88 (6-120)	0.000
	Serum ferritin		6-500 ng/ml	190.35 (7.2-697)	646.18 (13.3-1000)	0.000
	CRP		<6 mg/dl	30.73	46.71	-
	IL-6		<7 pg/ml	35.62 (0.19-385.2)	28.6 (2.6-186.5)	0.510

Mean age of the patients in non-severe category was 41 years ranging from 10 to 59 years while the mean age of the patients in the severe category was 44 years ranging from 13 to 59 years (Table 1).

Overall male to female ratio was 4:1 emphasizing that proportionately males were affected more than females in all the categories.

Hematological parameter taken into consideration was **NLR** derived by dividing percentage of neutrophils to percentage of lymphocytes after calculating the differential leucocyte count. Upper normal limit of NLR in adult population taken into consideration by us is 3.5, that given by Forget et al. [3]

Mean value of NLR in non-severe category was 3.7, almost half of the NLR mean values observed in severe category i.e., 6.7 (Table 1). On applying ANOVA 't' test, significant statistical correlation (p=0.016) of NLR was found with clinical severity.

Considering normal upper limit of NLR as 3.5, majority of patients (73.5%) in non-severe category had NLR < 3.5 as compared to severe category where majority had NLR ≥ 3.5 (66%).

Cytokine IL-6 is an inflammatory cytokine that has a role in different pathological conditions like infections, inflammations and in cancers. IL-6 is also considered as the main culprit responsible for hyperinflammation causing lung damage and eventually death in severe cases of COVID-19. Mean IL-6 values were 35.6 pg/ml in non-severe category while it was 28.6 pg/ml in severe category. On applying ANOVA 't' test for strength of significance in between groups, no significant association was found between IL-6 levels and clinical severity (p value = 0.510). Considering normal upper limit of IL-6 as 7pg/ml, in non-severe category 63.2% (43/68) patients had high IL- 6. Whereas 75% (24/32) patients had IL-6 value ≥ 7pg/ml in severe category.

Inflammatory parameters studied included **Serum Ferritin, ESR and CRP**

Ferritin is the major intracellular iron storage protein. It is an acute phase protein which is elevated in many inflammatory conditions, including acute infections. Higher serum ferritin values were found in severe category with mean of 646.1 ng/ml as compared to non-severe group with mean value of 190.3 ng/ml (Table 1). On applying ANOVA 't' test, significant statistical correlation (p=0.000) of serum ferritin was found with clinical severity. We

have taken normal upper limit of serum ferritin as 500 ng/ml considering the criteria of kit used in our laboratory. Majority of patients 95.5% (65 /68) patients in non-severe category had serum ferritin levels lower than 500 ng/ml, as compared to severe category where majority i.e., 65.6% (21/32) had serum ferritin more than 500 ng/ml.

ESR is a common hematology test which is sensitive but not so specific measure of inflammation. [4] Different normal range of ESR has been quoted in literature by different authors. For this study, we have considered normal upper limit as 20mm/hr for patients under 60 years of age. [5] Higher ESR values were seen in severe category with a mean value of 45.8 mm/hr while the non-severe category had a mean value of 24.2 mm/hr. On applying ANOVA 't' test, significant statistical correlation (p=0.000) of ESR was found with clinical severity. Majority (54.4%) had an ESR less than 20 mm/hr in the non-severe category. In the severe category, 68.7% patients had an ESR greater than or equal to 20 mm/hr.

For CRP in non-severe category, considering 6mg/l as the normal upper limit, only 29.4% had higher CRP levels with mean value of 30.7mg/l. While in severe category, majority 62.5% had high CRP with mean value of 46.7mg/l indicating that CRP levels rise with severity of disease.

Discussion:-

A total of 100 patients were included in this study. 68% belonged to non-severe category and 32% were placed in severe category. The mean age of COVID-19 cases was 41 years in non-severe category and 44 years in severe category. There was no significant difference in age in between the two groups. Overall male to female ratio was 4:1. In severe category, male to female ratio was 7:1 signaling that males are more likely to progress to severe disease and have COVID related complications as compared to females.

Hematological parameter studied was **NLR** which is a sensitive but less specific parameter to measure the severity of infection/inflammation. [6] Significantly high NLR was found in severe category (Mean=6.7) as compared to non-severe category (Mean=3.7). Our findings corroborated with that of another Indian study done by Singh et al [7]. In their study, mean NLR in severe cases came out to be 10.1 which is higher than mean NLR of severe category in our study. In a study conducted by Qin et al [8], it was noted that NLR was higher in severe category as compared to non-severe category (5.5 vs 3.2).

The results of our study are in concordance with the studies done by Zahorec et al., [6] Singh et al., [7] Yang et al., [9] and Wei et al., [10]. In all these studies, mean NLR in non-severe category was less than 4.5 except in the study done by Yang et al [9]. Similarly, in all studies, NLR in severe category was more than 5 except the study done by Wei et al [10]. In all of them, high NLR was found to be associated with severe category.

It can therefore be safely concluded that NLR can be used as an independent marker for predicting severity of COVID – 19. Further, cut-off value of 5 can be proposed to triage patients requiring intensive care (Table 2).

Table 2:- Comparison of NLR with different studies.

	Non-severe category	Severe category
Our study	3.7	6.7
Singh et al [7]	4.4	10.1
Qin et al [8]	3.2	5.5
Wei et al [10]	3.1	4.8
Yang et al [9]	4.8	20.7
Zahorec et al [6]	3.2	9.4

Several studies undertaken after COVID-19 was declared a pandemic have critically analyzed the role of inflammatory **cytokine IL-6** emphasizing its role in causing lung damage and mortality in severe cases of COVID-19 [8,11,12]. Based on this assumption, almost all leading health care facilities are resorting to IL-6 receptor antagonist therapy (tocilizumab) as a part of treatment protocol in moderate to severe cases. [13]

Several studies done nationally and internationally were compared with our results. The findings of our study are compatible with the results observed by Bhandari et al,[14] in their case 43.4% with raised IL-6 levels were falling in non-severe category as compared to our 63% i.e., significant proportion of non-severe patients who were

asymptomatic or had mild to moderate symptoms had raised IL-6 levels in both our and above-mentioned study conducted in Indian scenario. Our findings fully corroborate with the findings of Bhandari et al in severe category as 75% and 88% of those enrolled in both studies respectively showed raised or high IL-6 levels.

Comparing international study undertaken by Liu et al [15] whichtook into account 140 COVID positive patients and categorized them into mild and severe category. Serum IL-6 levels were then compared between the two clinical groups. It was observed that significant proportion (58.9%) of patients falling in mild category had raised IL-6 levels. Our data is in sync with observation made by Liu et al as 63.2% of our non-severe category patients also showed raised IL-6 levels. So, to conclude, serum IL-6 levels done at the time of admission failed to predict severity in COVID patients. This finding supports the dubious nature of IL-6 receptor antagonist in treating severe COVID as some of the studies done to assess efficacy of IL-6 receptor antagonist to treat moderate to severe cases failed to show expected results.

Serum ferritin is iron storage protein that is frequently measured as an indicator of iron storage in case of nutritional anaemia but it is also a well-known inflammatory marker. The levels of serum ferritin can increase significantly in response to inflammation and various diseases. Some of the studies conducted during COVID pandemic have concluded that serum ferritin levels can be used as an independent risk factor for disease severity in COVID-19 patients. Our study shows closer results to study conducted by Lin et al.,[16] as the mean serum ferritin values were comparable in non-severe (190/296 ng/ml) and severe categories (646/733ng/ml) respectively.

In the study conducted by Dahan et al.,[17] mean serum ferritin levels were considerably higher in both severe (2817/646ng/ml) and non-severe category (708/190 ng/ml) as compared to ours. Since the study conducted by Dahan et al., was in Israel which is a developed country and our study was conducted in a tertiary care center catering to rural population who are most of the time deficient in iron affecting serum ferritin levels. So, the levels observed at the time of COVID induced inflammation were not as high as observed by them. Our study as well as the two studies mentioned above indicate that higher serum ferritin levels can predict increased risk of disease severity in patients with COVID-19.

Although **ESR** is an indirect marker for inflammation, multiple studies conducted during COVID have shown that ESR levels can predict the severity of illness.One Indian study by Pujani et al., [18] that took into account ESR values in non-severe and severe cases as done by us shows comparative results. In their study, mean ESR values were 22.8 and 26.9 mm/hr respectively whereas in our study they were 24.3 and 45.9 mm/hr respectively in non-severe and severe category of patients. Our study showed comparatively higher elevation of ESR in severe cases as compared to the study mentioned above.

CRP is a non-specific acute phase reactant protein synthesized by hepatocytes under the influence of IL-6 and is considered a sensitive biomarker of inflammation, infection, and tissue damage. CRP levels are generally undetectable (<6mg/dl) in previously healthy individuals but it shows acute rise in trend during acute inflammatory responses. [15]

Our findings are in concordance with that of Qin et al [8] who took into consideration 452 COVID positive patients and calculated mean value of 33.2 mg/dl in non-severe category and 57.9 mg/dl in severe category. In our study, the values were 30.7 and 46.7 mg/dl respectively. However, study conducted in Indian scenario by Singh et al. showed almost thrice the elevation in mean CRP levels as compared to our study (non-severe = 92.5 mg/dl and severe = 239.4 mg/dl). This disparity may be because our study used a semi-quantitative test kit while they probably used a more sensitive and specific method than ours. Our study, the study mentioned above and various meta-analyses have all pointed out towards the fact that severe disease is associated with high CRP levels.

Thus, taking into account all the three non-specific markers of inflammation (acute phase reactants i.e., CRP, ESR, serum ferritin), our findings prove the point already highlighted by research studies done in India and abroad that severe disease in COVID-19 patients is associated with higher serum levels of all these biomarkers.

Conclusions:-

This is a retrospective study with hundred COVID positive patients in Sharda Hospital, SMS&R, Greater Noida during the first phase of COVID-19 pandemic. Patients were analyzed for hematological parameter - NLR and

inflammatory biomarkers like IL-6, CRP, ESR and ferritin according to their clinical severity i.e., mild, moderate, and severe category.

For the ease of comparison and to effectively analyze the results, mild and moderate categories were clubbed together into non-severe category and then compared with those placed in severe category at the time of admission. 68% of patients belonged to non-severe category (mild + moderate) and 32% were placed in severe category. Males are more likely to progress to severe COVID disease and have more COVID related complications as compared to females.

NLR can be used as an independent predictor for predicting severity of COVID-19. A cut off value of NLR < 5 can be used to segregate those patients requiring intensive care enabling judicious use of resources.

Serum IL-6 levels failed to predict severity of disease as majority of patients in mild to moderate category (non-severe disease) were having elevated IL-6 levels.

Considering all the three non-specific markers of inflammation (acute phase reactants i.e., CRP, ESR, Serum ferritin), our study concluded that severe disease in COVID-19 patients is associated with higher serum levels of all these biomarkers.

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