An Unknown Peak in Hemoglobin Chromatogram associated with Diabetic Markers and Oxygen Saturation in COVID-19 Patients

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Abstract

Coronavirus disease-19 (COVID-19) is associated with glycemic control, oxygen requirements and mortality among patients. Considering the vital role of hemoglobin in oxygen transport, this study focused on the effect of COVID-19 and diabetes on hemoglobin chromatogram patterns, especially in the context of inflammatory markers (IL6, hs-CRP, LDH and ferritin) and oxygen saturation. The study group (n= 242) was divided into four subgroups: COVID-19 patients (n=120; diabetics and non-diabetics) and non-COVID-19 controls (n=122; diabetics and non-diabetics). Hb chromatograms were obtained and analyzed using ion-exchange high-pressure liquid chromatography. All routine hematological and inflammatory markers were estimated in the samples drawn at admission.

The total monocyte count and inflammatory marker levels were highest among diabetic patients in the COVID subgroup ($p<0.0001$). An unknown peak (retention time 0.33-0.36) was observed in the hemoglobin chromatogram of patients with diabetes (both COVID-19 and non-COVID-19 subgroups). These patients with novel peaks presented significantly lower SpO2 levels at admission than those without diabetes but without this peak ($p=0.07$) and those without diabetes and peak ($p=0.001$) among the COVID-19 patients. The COVID-19 patients with this peak showed significantly poor glycemic control [HbA1c, fasting blood sugar and insulin sensitivity (QUICKI) ($p<0.05$)]. The area under the unknown peak (UNA) showed a positive linear correlation with HbA1b ($r=0.801$; $p<0.0001$), HbA1c ($r=0.65$, $p>0.0001$) and FBS ($r=0.56$, $p>0.0001$). This study reveals that COVID-19 patients with unknown peaks in the Hb chromatogram had higher inflammation, poor glycemic control and a significant need for oxygen.

Keywords: HbA1c, Haemoglobin, HPLC, SpO_2, Diabetic Mellitus, COVID-19.

Introduction

Diabetes affects coronavirus disease -19 (COVID-19) in two ways: individuals with diabetes are more predisposed to severe COVID-19 outcomes and COVID-19 increases the risk of developing diabetes[29].

Chen et al[5] linked COVID-19 to fasting blood sugar (FBS) and HbA1c levels, with higher levels in patients with severe COVID-19 than in those with mild COVID-19. Subsequently, increased blood glucose levels are associated with chronic inflammation and the release of inflammatory cytokines[14].

Glycated hemoglobin (HbA1c) is the gold standard for monitoring blood glucose levels and offers a three-month average value. A high percentage of HbA1c is associated with an increased risk of diabetic complications[24].

However, it is unknown whether an increase in HbA1c levels is associated with increased inflammation, oxygen saturation and mortality in diabetic patients with COVID-19 as compared to diabetic patients without COVID-19.

Previous studies have reported virus-induced hemoglobin denaturation and viral inhibition of heme metabolism by attaching to hemoglobin beta chains via surface glycoproteins. The viral alteration in hemoglobin may cause the SARS-CoV-2-caused syndrome of oxygen deprivation with multiple manifestations[18,37]. This hemoglobin alteration contributes to the complex sickness caused by oxygen deprivation. Hemoglobinopathy and iron dysmetabolism can significantly reduce the ability of erythrocytes to transport oxygen[37].

It has been shown that the virus may interact with hemoglobin molecules by way of ACE2, CD147, CD26 and possibly other receptors on erythrocytes and/or blood cell precursors[39]. This finding was facilitated by the fact that these receptors can be found in blood cell precursors. Previous studies have shown that the viral ORF8 protein and surface glycoprotein would attach to porphyrin and assault the heme located on the 1-beta chain of hemoglobin.

As SARS-CoV-2 promotes hemolysis and/or forms a compound with the released heme, the resultant hemoglobin is dysfunctional, resulting in diminished oxygen and carbon dioxide transport.

Lactate dehydrogenase (LDH) is an accurate indicator of hemolysis and LDH levels increase by two-to three-fold causing a decrease in hemoglobin[10,37]. Furthermore, ferritin regulates the immune response under conditions of ongoing inflammation by increasing anti-inflammatory cytokines

and reducing free radical damage[13]. This suggests that ferritin may be causally associated with inflammatory diseases. Hepatocytes are the main cells that are stimulated by the transcription of the C-reactive protein (CRP) gene in response to increased levels of inflammatory cytokines, particularly IL-6. Similar to ferritin, there is evidence that CRP functions as a crucial regulator and marker of inflammatory processes[33].

Estimating HbA1c can be achieved in a plethora of different ways including immunoassay, enzymatic assay, capillary electrophoresis, isoelectric focusing, ion-exchange high-performance liquid chromatography (HPLC), boronate-affinity chromatography and high-performance liquid chromatography (HPLC). Ion-exchange high-performance liquid chromatography and capillary electrophoresis are two of these that provide the benefit of graphical display of each run [15].

Based on the above context, this study focused on the observation of hemoglobin chromatogram patterns HbA1c among different subgroups based on COVID-19 cases (diabetics and non-diabetics) and non-COVID-19 controls (diabetics and non-diabetics), especially in the context of inflammatory markers (IL6, hs-CRP, LDH and ferritin) levels and oxygen saturation.

Material and Methods

Study population and their evaluation: This single-center observational study included 242 subjects:120 patients with confirmed COVID-19 and 122 age, sex and diabetes matched controls. RT-PCR of a nasopharyngeal sample was used to determine the SARS-CoV-2 infection status.

Experimental group: According to the COVID-19 status and FBS levels, the study subjects were sub-grouped into:
Group-A: Diabetic-COVID-19
Group-B: Non-Diabetic-COVID-19
Group-C: Diabetic Non-COVID-19
Group-D: Non-Diabetic Non-COVID-19

Clinical information: Diabetes was diagnosed using the WHO (2019) diabetes diagnostic criteria[9]. Subsequently, COVID-19 cases were stratified into the following categories: mild, oxygen saturation (SpO_2) 90–95%; moderate, SpO_2 75–89%; severe SpO_2 <75% as per the Indian Council of Medical Research (ICMR) guidelines[22].

Relevant data were collected from the patients including demographics, medical history, hematological findings and SpO_2 during admission. The ethics committee of the Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow (Dr. RMLIMS) approved the study protocol (IEC-66/2020).

Estimation of diabetic components and inflammatory markers: Serum C-peptide, cortisol, IL-6 and ferritin levels were determined using electrochemiluminescence on a Cobas 6000 automated analyzer (Roche Diagnostics). hs-CRP and LDH levels were evaluated using a Beckman Coulter AU 480 biochemical autoanalyzer (AU480).

The Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as follows:

QUICKI = 1/log (fasting insulin) + log (fasting glucose)[36]

Analysis of hemoglobin chromatogram by HPLC: HbA1c, HbA2 and HbF levels were determined using the ion-exchange-high-pressure liquid chromatography (HPLC) technique with the dual extended (HbA2/F/A1c) algorithm (Bio-Rad D10 Laboratories**,** Hercules, California, USA).

We selected the HbA1c expanded platform which detects variations in HbA1c, HbA2, HbF and other hemoglobin levels. The 6.5-minute extended approach is for better variant resolution. It is also the preferred method for detecting hemoglobin variations (Fig. 1A and B). In this experiment, HbF retention time was 0.41(0.41-0.46). According to the manufacturer's literature, HbF levels ≤ 10% do not affect HbA1c measurements[17].

HPLC was used to examine the hemoglobin chromatograms blindly without knowledge of the case/control status to ensure quality control. A case-control study was conducted using a 15% random sample and all results were reproduced with 100% accuracy.

Data analysis: Categorical variables were expressed as frequencies and percentages and compared using the chi-square test. As the distribution of inflammatory parameters was non-parametric across the different categories, all variables were expressed as medians (Q1; 25th percentile-Q3; 75th percentile). Inter-correlations among variables were analyzed using Pearson's correlation coefficient. Statistical significance was set at $P < 0.05$. SPSS Statistics for Windows, version 21.0 was used for all statistical analyses (IBM, USA).

Results

Demographical characteristics of the COVID-19 cases and controls: The demographic characteristics of the study groups including age, sex, severity and mortality parameter distributions, are shown in table 1. There was no significant difference in age and sex between controls and patients, suggesting adequate matching (p=0.957 and p=0.594, respectively). Of the 120 COVID-19 cases, the diabetic subgroup (A) had 30 (37.9%) severe, 23(29.2%) moderate and 26 (32.9%) mild cases, whereas the non-diabetic subgroup (B) had frequencies of 22.0%, 19.5% and 58.5% in the severe, moderate and mild categories respectively. Mortality was significantly higher (p=0.017) in the COVID-19 group with diabetes (n=19) than in the non-diabetic COVID-19 subgroup (n=02).

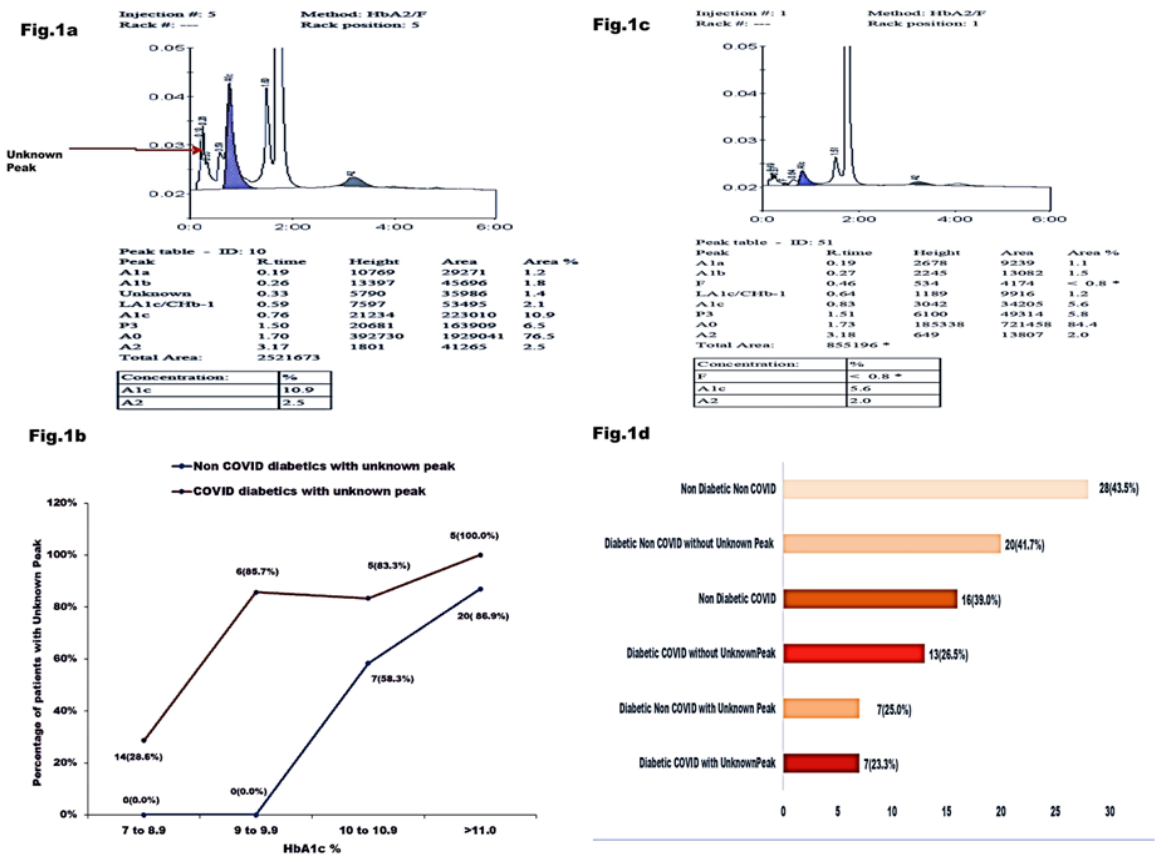


Fig. 1: (a) Showing the % of glycosylated hemoglobin (HbA1c) with an unknown peak (retention time 0.33-0.36) in COVID-19 patients with diabetes. (b) Showing an unknown peak as per the % of glycosylated hemoglobin (HbA1c) in COVID-19 patients with diabetes and Non-COVID-19 controls with diabetes (c) Showing the % of HbF in Non-COVID-19 without diabetes. (d) Showing the frequency of Fetal Hemoglobin (HbF%) in COVID-19 patients with or without an unknown peak (retention time 0.33-0.36).

Table 1
Demographical assessment in COVID-19 patients and the non-COVID-19 controls with and without Diabetic mellitus subjects

Variable		Cases		Controls		
		Diabetic COVID (A) (n=79)	Non-Diabetic COVID (B) (n=41)	Diabetic Non-COVID (C) (n=76)	Non-Diabetic Non-COVID (D) (n=46)	p-value
Age (Years)	Young (16-30 Yrs)	14 (17.7%)	10 (24.4%)	17(22.4%)	11(23.9%)	0.957
	Middle (31–45 Yrs)	21 (26.6%)	12 (29.3%)	21(27.6%)	13(28.3%)	
	Older (46-72 Yrs)	44 (55.7%)	19 (46.3%)	38(50%)	22(47.8%)	
Gender	Male	53 (67.1%)	29 (70.7%)	45(59.2%)	29(63.0%)	0.594
	Female	26 (32.9%)	12 (29.3%)	31(40.8%)	17(36.9%)	
Severity	Mild	26 (32.9%)	24 (58.5)	-	-	0.025*
	Moderate	23 (29.2%)	8 (19.5)	-	-	
	Severe	30 (37.9%)	9 (22.0)	-	-	
Mortality	Yes	19 (24.1%)	2 (4.9%)	-	-	0.017*
	No	60 (75.9%)	39 (95.1%)	-	-	
Unknown Peak	Yes	30 (37.9%)	-	28 (36.8%)	-	
	No	49 (62.1%)	-	48 (63.2%)	-	

The Chi-Square test was performed to determine the p-value. *p-value- <0.05 is considered statistically significant

Hematological, diabetic and inflammatory markers in COVID-19 and non-COVID-19 patients with and without diabetes: As shown in table 2, neutrophils (median 79.0; Q1:67.5-Q3:88.0) and monocytes (median 9.9; Q1:6.1-Q3:13.9) were the highest among diabetic patients in the COVID-19 subgroup (A) whereas lymphocyte count (median 14.0; Q1:7.0-Q3:23.0) was the lowest in the same subgroup. White blood cell (WBC) count was significantly higher in diabetic patients with COVID-19 (A: median 8.7; Q1:5.6-Q3:11.6) and diabetic non-COVID-19 (C; median 7.9; Q1:6.3-Q3:10.6) subgroups than in non-diabetic patients with COVID-19 (B: median 5.7; Q1:4.2-Q3:8.0) and without diabetes (D; median 5.8; Q1:5.5-Q3:6.9).

Table 2
Hematological, Diabetic and Inflammatory parameters assessment in COVID-19 patients and the non-COVID-19 controls with and without Diabetes mellitus

Variables	Cases		Controls						
	(A) Diabetic COVID-19 (n=79)	(B) Non-Diabetic COVID (n=41)	(C) Diabetic Non-COVID (n=76)	(D) Non-Diabetic Non-COVID (n=46)	p-value (A vs. D)	p-value (B vs. D)	p-value (C vs. D)	p-value (A vs. B)	p-value (A vs. C)
Hematological Parameters									
Hb (g/dL)	12.2 (10.9-13.2	12.7 (11.1-14.2)	12.6 (11.9-13.6)	12.7 (12.0-13.1)	0.085	0.834	0.803	0.150	0.133
Hct (%)	37.5 (33.7-41.3)	38.8 (34.6-42.9)	39.5 (35.9-42.7)	39.8 (38.5-41.4)	0.016*	0.455	0.384	0.299	0.259
RBCs ($\times 10^6/\mu L$)	4.4 (3.9-4.9)	4.5 (3.8-5.0)	4.6 (4.2-4.8)	4.5 (4.2-4.9)	0.403	0.556	0.862	0.925	0.297
MCV (fL)	88.8 (83.0-92.6)	88.9 (83.2-92.1)	88.2 (83.9-94.4)	90.3 (87.3-92.9)	0.100	0.363	0.374	0.437	0.341
MCH (pg)	28.8 (27.0-30.3)	29.0 (26.9-30.1)	28.6 (27.5-31.1)	28.8 (27.6-29.5)	0.815	0.811	0.427	0.826	0.466
MCHC (%)	32.2 (31.3-33.3)	32.1 (31.5-33.4)	32.1 (31.0-32.8)	31.8 (31.0-32.6)	0.094	0.235	0.711	0.909	0.146
WBC ($\times 10^3/\mu L$)	8.7 (5.6-11.6)	5.7 (4.2-8.0)	7.9 (6.3-10.6)	5.8 (5.5-6.9)	<0.0001*	0.168	<0.0001*	<0.0001*	0.951
Neutrophil (%)	79.0 (67.5-88.0)	61.0 (50.0-76.0)	66.0 (56.0-75.0)	62.0 (49.0-67.0)	<0.0001*	0.553	0.052	<0.0001*	<0.0001*
Lymphocyte (%)	14.0 (7.0-23.0)	25.5 (13.5-36.5)	25.0 (18.0-32.0)	28.0 (24.0-40.0)	<0.0001*	0.033*	0.036*	0.001*	<0.0001*
Monocyte (%)	9.9 (6.1-13.9)	9.5 (6.0-13.5)	5.0 (4.0-7.0)	6.0 (5.0-7.0)	<0.0001*	<0.0001*	0.851	0.751	0.775
PLT ($\times 10^3/\mu L$)	192.5 (129.0-254.0)	188.0 (131.0-228.0)	155.0 (135.0-183.0)	154.0 (119.0-216.0)	0.144	0.253	0.734	0.575	0.120
Metabolic Parameters									
HbA1c (%)	9.9 (7.6-11.4)	5.6 (5.3-5.8)	7.5 (6.6-8.8)	5.3 (4.9-5.6)	<0.0001*	0.004*	<0.0001*	<0.0001*	<0.0001*
HbA2 (%)	2.5 (2.3-2.9)	2.9 (2.5-3.2)	2.4 (2.2-2.9)	2.7 (2.6-2.9)	0.014*	0.212	0.023*	0.012*	0.946
FBS (mg/dL)	196.0 (146.0-252.0)	105.0 (101.0-110.0)	170.0 (140.0-208.0)	100.0 (92.0-107.0)	<0.0001*	0.018*	<0.0001*	<0.0001*	0.168
Inflammatory Parameters									
LDH(U/L)	351.5 (222.0-503.0)	237.5 (163.5-283.5)	196.0 (162.5-220.4)	188.0 (161.0-210.0)	<0.0001*	0.034*	0.099	<0.0001*	<0.0001*
IL-6 (pg/ml)	33.2 (11.2-80.1)	13.3 (6.1-35.4)	5.4 (3.9-9.2)	5.3 (3.6-8.7)	<0.0001*	<0.0001*	0.274	0.002*	<0.0001*
hs-CRP (mg/L)	8.5 (3.0-17.0)	2.0 (1.0-5.4)	1.3 (1.0-2.8)	1.6 (1.1-2.7)	<0.0001*	0.251	0.596	<0.0001*	<0.0001*
Ferritin (ng/mL)	477.0 (181.0-989.5)	129.0 (57.0-490.5)	101.0 (68.0-208.0)	100.0 (64.0-141.0)	<0.0001*	0.072	0.510	0.004*	<0.0001*

Abbreviation- Q-quartile, Hb-Hemoglobin, Hct- Hematocrit, RBCs- Red Blood Cells, MCV-Mean corpuscular volume, MCH-Mean corpuscular hemoglobin, MCHC- Mean corpuscular hemoglobin concentration, WBC- White Blood Cells, PLT- Platelets, HbA1c- Hemoglobin A1C, HbA2-Hemoglobin A2, FBS- Fasting Blood Sugar, LDH- Lactate Dehydrogenase, IL-6 – Interleukin-6, hs-CRP- High-Sensitivity C-reactive Protein. *p-value- <0.05 is considered statistically significant.

HbA2 was significantly decreased among diabetics with COVID-19 (A; median 2.5; Q1:2.3-Q3:2.9) as well as in diabetic non-COVID (C; median 2.4; Q1:2.2-Q3:2.9) subgroups in comparison to non-diabetic with COVID-19 (B; median 2.9; Q1:2.5-Q3:3.2) and without diabetes (D; median 2.7; Q1:2.6-Q3:2.9) while HbA1c and fasting blood sugar level were significantly higher among diabetics ($p<0.0001$) when compared to non-diabetics.

The levels of all inflammatory markers (IL6, hs-CRP, LDH and ferritin) were higher in the COVID-19 group than in the control group. Furthermore, the presence of diabetes among COVID was synergistic and led to the highest level ($p<0.0001$ in comparison to all other subgroups) of all of these markers i.e. IL6 (median 33.2; Q1:11.2-Q3:80.1), hs-CRP (median 8.5; Q1:3.0-Q3:17.0), LDH (median 351.5; Q1:222.0-Q3:503.0) and ferritin (median 477.0; Q1:181.0-Q3:989.5) (Table 2).

FBS and HbA1c levels showed a weak negative correlation with monocytes (r=-0.27, p=0.003 and r=-0.26, p=0.004, respectively). Similarly, LDH levels were negatively correlated with SpO2 levels (r=-0.27, p=0.003). Interestingly, HbA1c was negatively correlated with SpO_2 in COVID-19 patients (r=-0.29, p=0.002). The hs-CRP and ferritin levels were significantly correlated with the HbA1c levels (Fig. 2). However, the other markers did not correlate with HbA1c% (data not shown).

Description of an unknown peak in hemoglobin chromatogram: The hemoglobin HPLC pattern showed that 25% (n=30) of the COVID-19 patients (cases; n=120) and 36.8% (n=28) of the non-COVID patients (controls; n=122) had an unknown peak at the retention time (0.33-0.36) (Table 1; Fig.1a). This peak was observed exclusively among patients with diabetes in both the COVID-19 and non-COVID-19 subgroups (Table 1). HbF was least frequent among COVID-19 diabetics with an unknown peak (23.3%; n=7) whereas it was most prevalent among non-COVID-19 non-diabetic patients (43.5%; n=28) (Fig. 1c and 1d).

HbA1b levels were significantly decreased ($p<0.0001$) among COVID-19 diabetics with an unknown peak in comparison to COVID-19 diabetics without an unknown peak (Table 3) and its concentration (area under HbA1b peak) was strongly correlated (r=0.801; $p<0.0001$) with the concentration of unknown peak (area under peak: UNA) (Fig.4). The concentration of the unknown peak (area under the peak: UNA) was moderately correlated with fasting blood sugar (FBS; r=0.56; $p<0.0001$) and HbA1c (r=0.65; $p<0.0001$) (Fig.4).

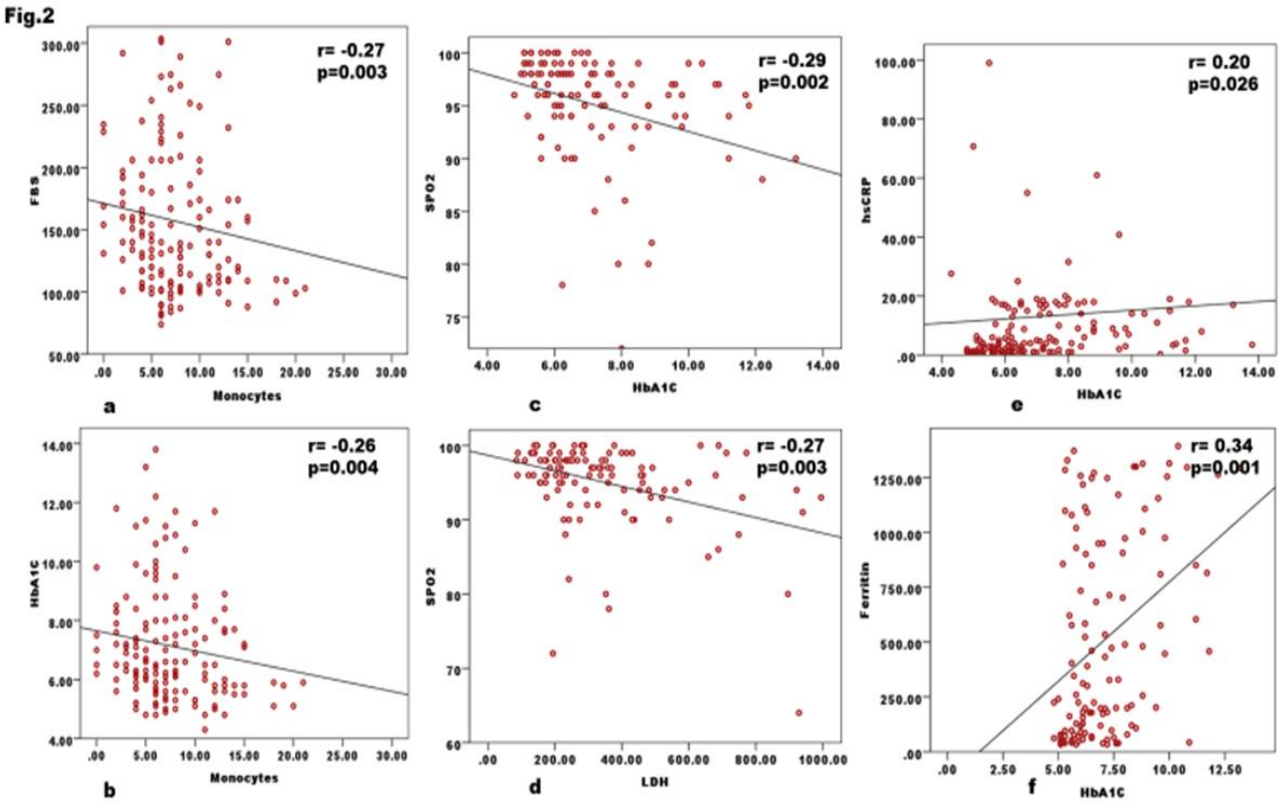


Fig. 2: Correlation between monocyte count and fasting blood sugar level (FBS). Correlation between oxygen saturation (SpO_2) and glycosylated hemoglobin (HbA1c). Correlation between Hs-CRP and glycosylated hemoglobin (HbA1c). Correlation between monocyte count and glycosylated hemoglobin (HbA1c). Correlation between oxygen saturation (SpO_2) and lactate dehydrogenase (LDH). Correlation between ferritin and glycosylated hemoglobin (HbA1c).

Table 3
Comparison between parameters with unknown peak and without an unknown peak in COVID-19 patients

Variables	Patients with Unknown peak (Retention Time between 0.33-0.36) Median (Q1-Q3); n=30	Patients without Unknown peak (Retention Time between 0.33-0.36) Median (Q1-Q3); n=90		p-value	p-value	p-value
	A) Diabetics with Unknown Peak (n=30)	B) Diabetics without Unknown Peak (n=49)	(C) Non-diabetics (n=41)	A vs. B	A vs. C	B vs. C
SpO2 Level	95.0(90.0-97.0)	96.0 (94.0-98.0)	98.0(97.0-99.0)	0.076	<0.0001*	0.001*
Metabolic parameters						
HbA1c (%)	9.5 (8.25-11.05)	7.1(6.5-7.7)	5.6(5.1-5.8)	0.001*	<0.0001*	<0.0001*
HbA1b RT	0.26 (0.26-0.27)	0.27 (0.27-0.27)	0.27 (0.27-0.27)	<0.0001*	<0.0001*	0.473
HbA1b Area (%)	1.20(1.00-1.40)	1.90(1.501-2.20)	1.40 (1.20-1.50)	<0.0001*	0.055	<0.0001*
FBS (mg/dL)	225.9 (195.9-270.4)	154.0 (134.0-174.0)	93.0 (89.0-100.0)	<0.0001*	<0.0001*	<0.0001*
QUICKI	0.29(0.26-0.32)	0.31(0.28-0.36)	0.31 (0.29-0.36)	0.043*	0.086	0.990
Iron (µg/dL)	32.0(26.5-47.5)	41.0(25.0-61.0)	56.0 (30.0-81.0)	0.259	0.007*	0.048*
C-Peptide (ng/ml)	1.3(0.59-3.49)	0.99(0.20-1.69)	0.88 (0.26-3.04)	0.115	0.542	0.341
Cortisol (µg/dL)	12.0(6.3-36.7)	12.4 (6.3-25.5)	13.3(5.9-23.7)	0.854	0.948	0.909
Inflammatory parameters						
IL-6 (pg/ml)	38.3(16.6-103.1)	14.4(6.4-44.3)	10.7(4.5-26.0)	0.020*	<0.0001*	0.045*
hs-CRP (mg/L)	11.0(5.5-17.0)	4.6(1.8-16.0)	2.0(1.1-5.7)	0.041*	<0.0001*	0.006*
LDH (U/L)	462.0(312.5-684.0)	233.0 (189.5-431.0)	209 (159.9-258.0)	0.048*	<0.0001*	0.001*
Ferritin(ng/mL)	702.0(251.0-1294.5)	209.0 (70.0-610.0)	132.0 (68.0-469.0)	0.001*	<0.0001*	0.042*

Abbreviation- SpO2- Oxygen saturation, RT- Retention Time, PLT- Platelets, RBCs count-Red Blood Cells, HbA1c- Hemoglobin A1c, HbA1b- Hemoglobin A1b, FBS- Fasting Blood Sugar, QUICKI-Quantitative Insulin Sensitivity Check Index, LDH- Lactate Dehydrogenase, IL-6-Interleukin-6, hs-CRP- High-Sensitivity C-reactive Protein. *p<0.05 is considered statistically significant.

Hemoglobin chromatogram pattern with an unknown peak (retention time between 0.33-0.36) associated with increased inflammatory markers: Table 3 presents the categorization based on the presence of unknown peaks and diabetes. All the inflammatory markers i.e. IL-6, hs-CRP, LDH and ferritin were multifold higher (p<0.0001) in COVID-19 diabetics with unknown peak (IL-6- median, 38.3, Q1 16.6- Q3 103.1; hs-CRP- median 11.0, Q1 5.50- Q3 17.0, LDH- median 462.0, Q1 312.5- Q3 684.0, ferritin- median 702.0, Q1 251.0 – Q3 1294.5) in comparison to COVID non-diabetics (IL-6- median, 10.7, Q1 4.5- Q3 26.0; hs-CRP- median 2.0, Q1 1.1 - Q3 5.7, LDH- median 209.0, Q1 159.9- Q3 258.0, ferritin- median 132.0, Q1 68.0 – Q3 469.0) as well as COVID diabetics without unknown peak (IL-6- median, 14.4, Q1 6.4- Q3 44.3; hs-CRP- median 4.6, Q1 1.8- Q3 16.0, LDH- median 233.0, Q1 189.5- Q3 431.0, ferritin- median 209.0, Q1 70.0 – Q3 610.0) (p<0.05). The levels of these markers among COVID-19 diabetics without unknown peaks were also higher (p<0.05) than those among non-diabetics.

Fig.5 shows that IL-6 levels were significantly higher in COVID-19 diabetics with an unknown peak group (median: 51.48; Q1 28.53-Q3 242.20) than in non-COVID-19 diabetic patients with an unknown peak group (median: 4.90; Q1 2.12- Q3 7.86) (p<0.0001) (Fig.5a). Similarly, LDH was also significantly increased in COVID-19 diabetics with an unknown peak (median 422.50; Q1 252.0- Q3 680.0) than in non-COVID-19 diabetes patients with an unknown peak (median 192.75; Q1 158.7- Q3 230.10) (p<0.0001) (Fig.5c). hs-CRP and ferritin levels were significantly higher in patients with COVID with an unknown peak (median 12.33; Q1 6.01- Q3 18.01 and median 860.5; Q1 458.0- Q3 1263.0) than in non-COVID-19 diabetics with an unknown peak (median 1.69; Q1 0.57- Q3 3.45; p=0.001and median 105.5; Q1 79.0 – Q3 174.95, p<0.0001) (Fig. 5 b and d).

Distribution of COVID-19 and non-COVID patients based on unknown peak (retention time between 0.33-0.36) based on HbA1c percentage: The frequency of an

unknown peak in COVID-19 patients with and without diabetes in relation to HbA1c % is shown in fig. 1b. In COVID-19 diabetic patients, an unknown peak was observed at 7.0 HbA1c% whereas in non-COVID diabetic patients, an unknown peak was observed at 9.0. With increasing HbA1c% (10-10.9), 83.3% of COVID-19 in patients with diabetes showed an unknown peak. However, at the same HbA1c % (10-10.9), only 58.5% of non-COVID-19 patients without diabetes had this unknown peak. Furthermore, this unknown peak was observed in 100% COVID-19 with diabetic patients at 11-13.3 HbA1c % while only 86.9% of non-COVID-19 without diabetic patients were with this unknown peak.

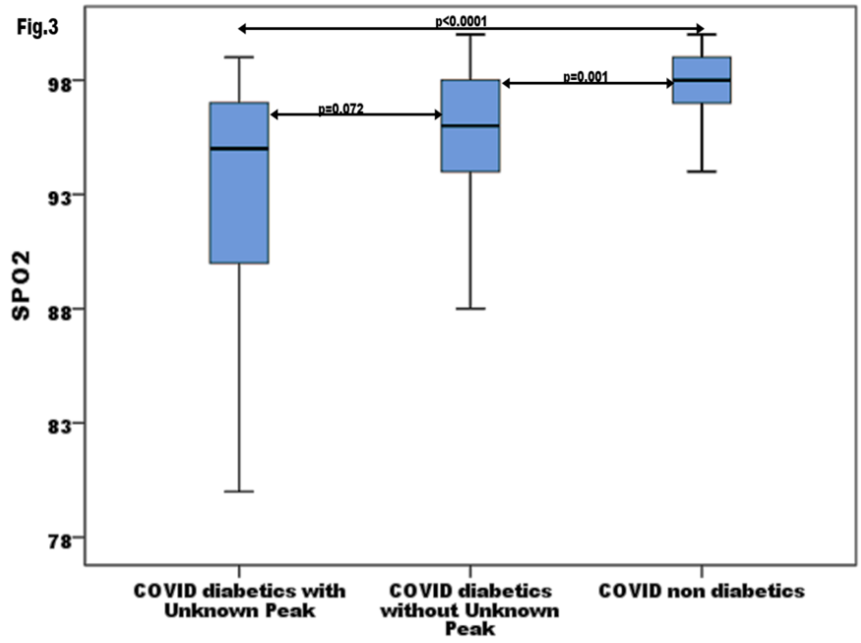


Fig. 3: Showing the status of Oxygen Saturation (SpO2) levels with an Unknown peak (retention time 0.33-0.36) in COVID-19 patients with and without diabetes.

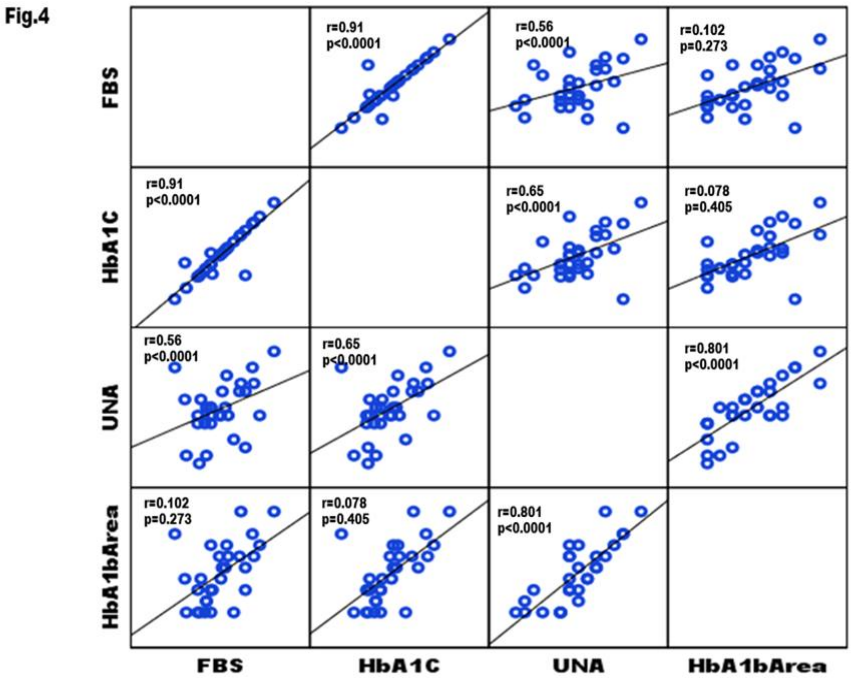


Fig. 4: Correlation between glycosylated hemoglobin (HbA1c), Hemoglobin A1b (HbA1b) and Fasting blood sugar (FBS) levels with the area percentage of the unknown peak (UNA).

Fig 5a

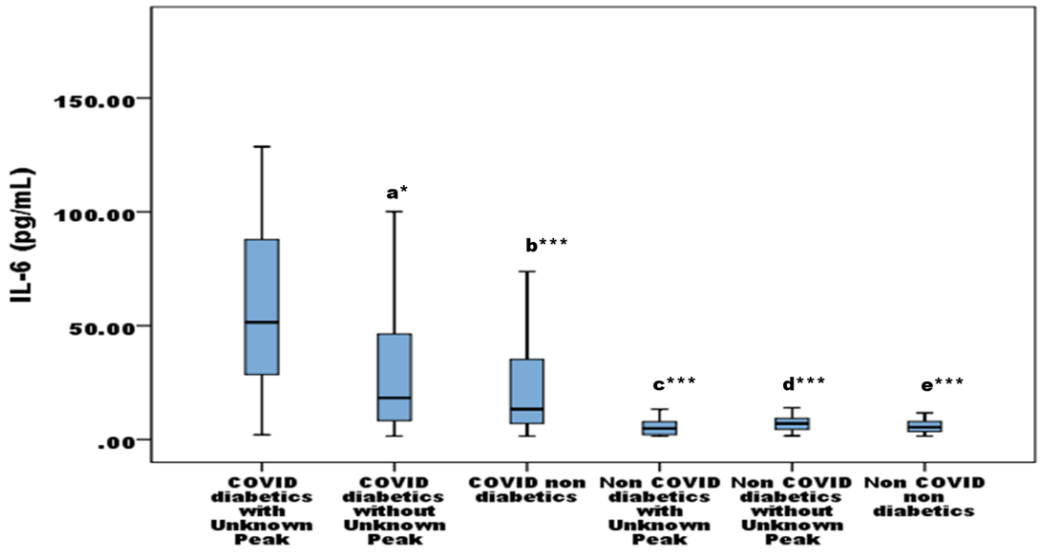


Fig 5b

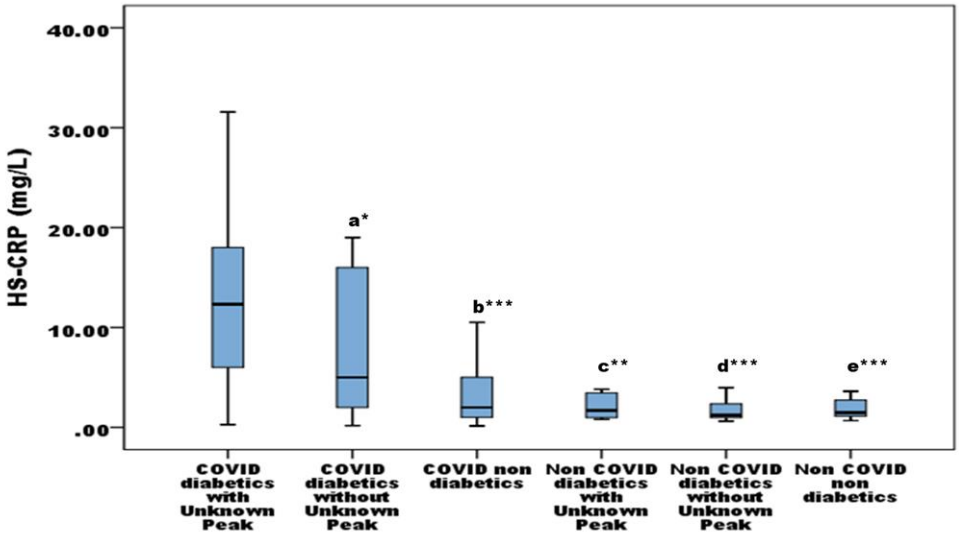
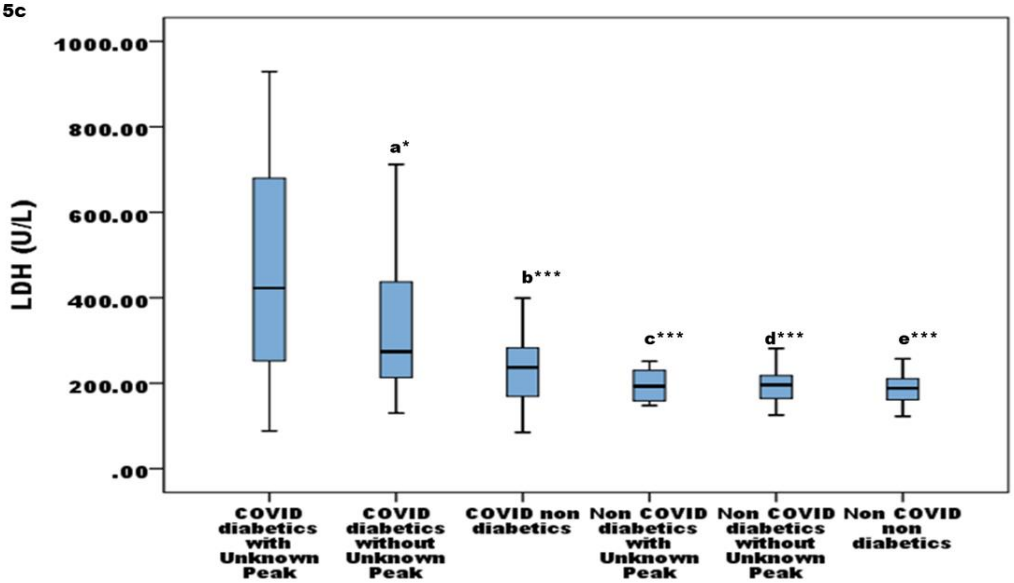


Fig 5c



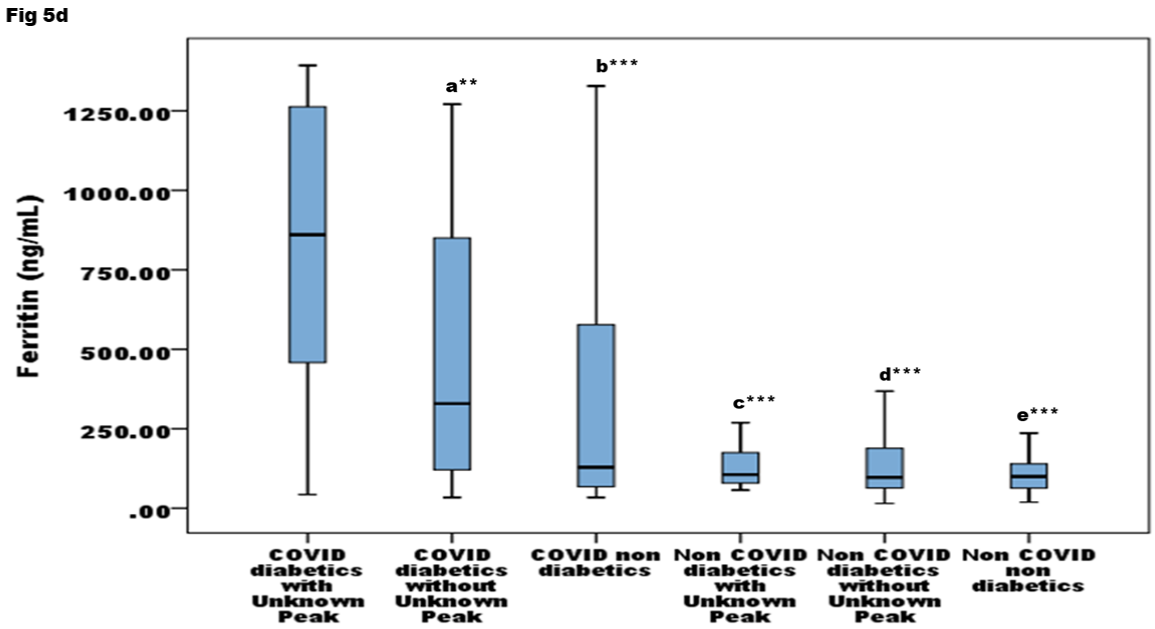


Fig. 5: Inflammatory parameters assessments in study population.
(5a) shows the status of Ferritin level, (5b) shows the LDH level status, (5c) shows the status of IL-6 levels and (5d) shows the status of hs-CRP levels.

Abbreviations: (a) P-value calculated between COVID diabetics with unknown peak and COVID diabetics without peak.
(b) P-value calculated between COVID diabetics with unknown peak and COVID non-diabetics.
(c) P-value calculated between COVID diabetics with unknown peak and non-COVID diabetics with band.
(d) P-value calculated between COVID diabetics with unknown peak and non-COVID diabetics without the band.
(e) P-value calculated between COVID diabetics with unknown peak and non-COVID non-diabetics.
*P value <0.05, **P-value 0.001, ***P-value <0.0001.

The frequencies of HbF were in non-diabetic non-COVID-19 patients 28(43.5%) followed by diabetic non-COVID without unknown peak >non-diabetic COVID-19> diabetic COVID-19 with unknown peak > diabetic non-COVID-19>diabetic COVID-19 with unknown peak (Fig.1d). However, an unknown peak was observed in 7(25%) diabetic patients, but the area was ≤10% of HbF that did not interfere with HbA1c (Fig. 1b). SpO2 levels were significantly associated with COVID-19 diabetes at unknown peaks in the non-diabetic group (median: 95 vs.97, p<0.0001) (Fig. 3).

Discussion

COVID-19 and diabetes have recently been linked, revealing complicated pathophysiological characteristics that underpin hyperglycemia and overall glucometabolic distress[2]. Although more pronounced, COVID-19 may cause an inflammatory process similar to that in T2DM[23,31]. Protracted islet hyperstimulation and glucose toxicity may cause beta cell depletion and worsening of diabetes[3,8]. In our study, white blood cell (WBC), neutrophil and monocyte counts were significantly increased, whereas lymphocyte counts were decreased in COVID-19 patients with diabetes.

Our results are consistent with those reported by Yuan et al[38] in severe COVID-19 patients. A study by Tan et al[35] involving 90 hospitalized COVID-19 patients reached the same conclusion. Increased total leukocyte and neutrophil counts are commonly observed in severe COVID-19 patients[32]. A meta-analysis showed that 62.5% of COVID-19 patients have lymphopenia[26]. It has been reported that low lymphocyte counts in severely ill patients may indicate a poor prognosis[35].

COVID-19-induced hyperinflammation is primarily mediated by neutrophils which boost cytokine production through degranulation[25]. SARS-CoV-2 infection of antigen-presenting cells (monocytes) directly inhibits adaptive antiviral immune response. After monocyte infection, systemic hyperinflammation may be controlled by inhibiting cytokine generation and cytokine-mediated signaling pathways. Our study results were consistent with these findings: lymphocyte and monocyte counts were higher in the diabetic- COVID-19 patients. The metabolic variables FBS, HbA1c, C-peptide and ferritin levels were significantly decreased in COVID-19-diabetes patients**.**

Furthermore, monocyte count was negatively correlated with HbA1c and FBS i.e. diabetes severity. Our data showed that the levels of the inflammatory markers LDH, IL-6, hs-CRP and ferritin were more than twice as high in patients with an unknown peak (severe diabetes due to COVID-19). These findings were consistent with those of COVID-19 studies that reported high levels of interleukin-6, C-reactive

protein (CRP) and fibrinogen, which are associated with inflammation have been linked to COVID-19 patients[16,28].

The study revealed that Inflammation is a vital factor in the progression of COVID-19 in T2D; however, low-grade chronic inflammation, typical in patients with diabetes, increases the risk of inflammatory storms, resulting in more severe cellular damage[1,6,20]. Another study demonstrated that IL-6, ferritin and CRP were elevated among COVID-19 patients[19].

Subsequently, the HPLC pattern of Hb chromatography showed that Hb1b and HbA1c levels were elevated during hyperglycemia. In COVID-19 with diabetic patients, HbA1b was slightly left-shifted on Hb chromatogram and an unknown peak (retention time: 0.33-0.36) was observed in all diabetic COVID patients and this unknown peak was not part of HbF. Furthermore, this unknown peak was observed earlier (at 7% HbA1c) whereas in non-COVID-19 diabetic patients, it was at >10% HbA1c. Patients with an unknown peak had significantly higher HbA1c, FBS, C-peptide and insulin sensitivity (QUICKI) levels than those without a peak. Moreover, rapidly rising blood glucose levels can be identified by simultaneous measurement of HbA1c and glycoalbumin levels.

The detection of an unknown peak in patients with relatively high HbA1c and inflammation in COVID-19-infected diabetics indicates that there might be some new substance that appears in the peak, possibly because adult human erythrocytes include some minor hemoglobins in addition to the major hemoglobin (HbA) (HbA1a, HbA1b and HbA1c). Non-enzymatic glycosylation of HbA may occur when phosphorylated hexoses (glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-biphosphate) and trioses (glyceraldehydes-3-phosphate, dihydroxyacetone phosphate) containing a free aldehyde or ketone group are present[34].

All hemoglobin A adducts were indistinguishable from HbA_{1b} by ion-exchange chromatography and isoelectric focusing. This indicated that either glucose-6-phosphate or its metabolite (glycolysis pathway) reacts with hemoglobin to form a subfraction of an unknown peak[34]. Iron levels decreased and ferritin levels increased in patients with unknown peaks (diabetic COVID). Nevertheless, inflammation-induced changes in iron homeostasis are significant clinical predictors of risk stratification in SARS-CoV-2-infected diabetic patients.

SARS CoV-2 effect on hemoglobin is a possible pathogenic mechanism[27] whereas COVID-19 is thought to be an acute acquired porphyria[4]. SARS-CoV interferes with hemoglobin at the erythrocyte level; the cytoplasmic/nuclear material of the virus facilitates virus replication and interacts with hemoglobin via CD147 and/or CD26. SARS-CoV-2 interaction with iron metabolism and oxygen supply could be attributed to phylogenetic mechanisms that evolved in oxygen-free and iron-rich ancestral environments: virus-induced iron dysmetabolism, ferroptosis and oxidative stress affect the host immune response and related proinflammatory pathways[7]. More significantly, in this investigation, patients with diabetes and COVID-19 had a threefold increase in LDH levels.

This conclusion was confirmed by Zhou et al[39] who observed LDH levels to increase by two to three times in severe cases, concurrent with a drop in hemoglobin[10].

Chen et al[6] observed that 63% of COVID-19 patients had elevated ferritin levels, which may be related to poor clinical outcomes. Consequently, hemoglobin concentrations were substantially associated solely with CRP levels and tended to be associated with IL-6 levels.

In addition, the CRP and IL-6 levels in individuals with functional iron insufficiency were significantly elevated. The correlation between ferritin, CRP and IL-6 levels was high. These findings are consistent with the link between abnormal iron homeostasis and progressive inflammation, which promotes lung damage and respiratory failure[21].

One interesting finding was that the unknown peak was evident at lower HbA 1c levels (7-10%) in certain COVID-19 patients in comparison to non-COVID-19 patients with an unknown peak (HbA1c>10%). Since QUICKI (Insulin Sensitivity) was lower in COVID-19 patients with unknown peaks, these patients suffered from insulin resistance. Thus, a higher influx of glucose into glycolysis leads to higher levels of intermediates and more glycation leads to an increase in HbA 1b and an unknown peak. All such cases of the earlier appearance of an unknown peak to lower HbA 1c level (7 -10 %) were associated with high fasting sugar levels, indicating a recent dysglycemia.

SpO2 was significantly associated with an unknown peak, HbA1c% and LDH, indicating that the unknown peak was due to severe uncontrolled hyperglycemia in patients with COVID-19 which was also associated with higher inflammation, leading to worse clinical outcomes.

Additionally, it may increase the affinity of hemoglobin to oxygen and hence may decrease the oxygen delivery to peripheral tissues, or the oxygen-carrying capacity of hemoglobin might have been reduced since SpO2 was significantly decreased among patients[12,30] who presented with an unknown peak and oxygen therapy was required for patients carrying an unknown peak. This favors the reduced oxygen-carrying capacity of hemoglobin.

This study has some limitations that should be addressed: a large sample population is required for significant statistical power and further studies are required to explore the metabolic pathways in patients with diabetes COVID-19 to establish how they differ from those in non-COVID-19 diabetic patients.

Conclusion

Individuals with diabetes and COVID-19 experience a multifold increase in the levels of metabolic and inflammatory markers. HbA1b and HbA1c levels are elevated during hyperglycemia and SARS-CoV-2 infection. In patients with severe diabetes, HbA1b slightly shifted left on the Hb chromatogram and an unknown peak with a retention time of 0.33-0.36 appeared in such patients. This unknown peak was highly related to COVID diabetic patients and significantly influenced iron, ferritin, LDH, IL-6, hs-CRP and oxygen saturation (SpO_2). Thus, this study implies that patients with T2DM are more prone to an inflammatory microenvironment which may lead to more severe respiratory infections and that both diabetes and COVID-19 may synergistically affect and aggravate impaired glucose metabolism.

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