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A novel metric-based approach of scoring early host immune response from oro-nasopharyngeal swabs predicts COVID-19 outcome

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Unpredictable fatal outcome of COVID-19 is attributed to dysregulated inflammation. Impaired early adaptive immune response leads to late-stage inflammatory outcome. The purpose of this study was to develop biomarkers for early detection of host immune impairment at first diagnosis from leftover RNA samples, which may in turn identify high risk patients. Leftover RNA samples of COVID-19 patients at first diagnosis were stored. Following prospective follow-up, the samples were shorted and categorized into outcome groups. Impaired adaptive T cell response (severity score) and Impaired IL-10 response (undetectable IL-10 in the presence of high expression of a representative interferon response gene) were determined by RT-PCR based assay. We demonstrate that a T cell response based 'severity score' comprising rational combination of Ct values of a target genes' signature can predict high risk noncomorbid potentially critical COVID-19 patients with a sensitivity of 91% (95% CI 58.7–99.8) and specificity of 92.6% (95% CI 75.7–99) (AUC:0.88). Although inclusion of comorbid patients reduced sensitivity to 77% (95% CI 54.6–92.2), the specificity was still 94% (95% CI 79.8–99.3) (AUC:0.82). The same for 'impaired IL-10 response' were little lower to predict high risk noncomorbid patients 64.2% (95% CI 35.1–87.2) and 82% (95% CI 65.5–93.2) respectively. Inclusion of comorbid patients drastically reduce sensitivity and specificity51.6% (95% CI 33.1–69.8) and 80.5% (95% CI 64.0–91.8) respectively. As best of our knowledge this is the first demonstration of a metric-based approach showing the 'severity score' as an indicator of early adoptive immune response, could be used as predictor of severe COVID-19 outcome at the time of first diagnosis using the same leftover swab RNA. The work flow could reduce expenditure and reporting time of the prognostic test for an earliest clinical decision ensuring possibility of early rational management.

Keywords COVID-19, Severity score, IL-10 response, Oro-nasopharyngeal swab, T cell response, Prognostic, Biomarker, Leftover RNA, High risk

The pandemic of coronavirus disease 2019 (COVID-19) was caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The manifestation of the infection varied grossly in the population. Many patients though initially present as asymptomatic or with mild symptoms, in later stage, develops severe respiratory and systemic symptoms due to deregulated inflammatory state called "cytokine storm" leaving little option for intervention[1–3]. Conventional clinical, radiological, thrombo-inflammatory blood biomarkers cannot predict the disease outcome precisely at the early stage before immune deregulation takes place[4,5] and when prophylactic

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management might be more effective. A battery of early host response biomarkers is required for risk assessment of patients at the earliest contact with the health care system.

Recently, Li et al. reported a method in which evaluation of 25 gene signatures in broncho alveolar lavage samples by single cell RNA sequencing can distinguish severe from mild COVID-19cases[6]. However clinical feasibility of implementing single cell technology is yet to be evaluated. Upregulation of inflammatory markers like calprotectin (S100A8, S100A9) and Galectin-3 released from neutrophils have been reported in severe COVID-19 along with other inflammatory markers in blood[7,8]. Calprotectin transcripts were also reported (S100A6, S100A8, S100A9 and S100P) in nasopharyngeal swab-derived RNA from infected patients[9,10], however clinical implementation for detecting severe cases is yet to be evaluated. A serum-based two marker scoring (IL-6 and IL-10) was reported to show sensitivity (51.4%) in detection of severe outcome (ICU/death)[11]. A SARS-CoV-2 unusual micro-RNA "miR-nsp3-3p" has been identified from patients serum predicting severe outcome[12], however it does not reflect actual host immune impairment. Recently Kazuko Uno et al. has reported identification of serum biomarkers to predict prognosis in COVID-19 patients[13].

Thus, most of the prognostic biomarkers for COVID-19 have been evaluated using inflammatory biomarkers either secreted serum/plasma factors or markers expressed in peripheral blood leukocytes. The neutrophil activation and tissue infiltration markers such as calprotectin[14] are also indicative of an already established deregulation of inflammatory response and may not be used as tools for early diagnostic for therapeutic intervention. The deregulated inflammation and cytokine storm start late in the disease when immune response fails to clear the infection in its early phase in upper respiratory tract. Moreover, the blood based biomarkers may not represent true early local immune response at the upper respiratory system. It has been recently shown that failed local anti viral immune response in nasal epithelium leads to severe form of COVID-19 infection[10]. Optimum immune homeostasis at the entry point may limit the infection locally. So it is important to develop oro-nasopharyngeal tissue-based prognostic biomarkers which could identify potential severe cases in early phase of the disease when most of the newer antivirus or any prophylactic measures may work more effectively. For this purpose, a prognostic biomarker development using leftover RNA of oro-nasopharyngeal swab samples following diagnosis of COVID-19 can be the best solution. Such an approach is also expected to reduce expenditure and may expedite the acquisition of data required for timely clinical intervention. Very few studies have been reported on prognostic biomarkers of COVID-19 using RNA derived from leftover oro-nasopharyngeal swab samples following SARS-CoV-2 diagnosis[9].

Initial response to viral infection is the interferon activation in the infected cells. Though type of interferon may vary depending on infected cell population, downstream signalling and activation of interferon stimulated genes (ISGs) share a common signature[15]. The expression of ISGs mediates cascade of antiviral innate and adaptive responses to limit the infection[15,16]. OAS group of ISGs (OAS1, OAS2 and OAS3) are activated in response to interferon and mediate viral RNA destruction[17]. In immune homeostasis, pro-inflammatory signalling is always balanced by anti-inflammatory response. One of the key mediators of anti-inflammatory arm of innate immunity is the interleukin 10 (IL-10). It is secreted from almost all of the immune cells including T cells and B cells as well as by some epithelial cells[18,19]. In mouse model, it has been shown that interferon response in absence of IL-10 leads to uncontrolled inflammation and spontaneous colitis, indicating the importance of IL-10 in mucosal immune homeostasis as an important regulatory mechanism to prevent uncontrolled inflammation[20–22]. Additionally, IL-10 also plays a role in modulating effective adaptive immunity and localization of infection following a virus infection[23,24]. Though, some studies on COVID-19 severe cases have reported upregulation of IL-10 and IL-6 both in blood and lung[25,26], they were conducted at a stage when the cytokine storm had already taken place with all the SOS mechanisms in blood activated at the highest level. Thus they may not represent the early impairment which takes place at the site of entry of the virus. No report is available on how the balance of interferon and IL-10 response may be altered at oro-nasopharyngeal mucosa at early state to produce a severe outcome.

Early activation of adaptive B cell and T cell mediated immunity is important to clear and localize the virus infection before systemic dissemination. Evidence shows that anti SARS-CoV-2T cell immunity has greater role in preventing severe form of COVID-19, and detection of anti-SARS Cov-2 specific T cells has been demonstrated ex vivo within 4 days following onset of symptoms[27,28]. Since dysregulated cytokine storm emerges when effective adaptive immunity fails to clear virus infection[29], it would be more feasible to use a biomarker which is based on T cell response. Identification of such a biomarker can serve as early prognostic marker and may have better prognostic value than other inflammatory markers which are currently in use or under investigation. Though importance of T cell mediated immunity in COVID-19 has been recognized, however present methods of analysing the virus specific T cell activation is very complex, expensive and time consuming[30]. Those methods can't be clinically applicable for a large population having acute health crisis.

To identify early prognostic marker/s, we investigated inflammatory and adaptive T cell response markers in the leftover RNA samples upon diagnosis of COVID-19 using RT-PCR, and evaluated their association with disease outcome. Our data demonstrate that a T cell response-based severity score can potentially identify high risk patients at the time of first diagnosis of COVID-19 infection without any of additional sampling/testing. Our approach of prognostic evaluation in leftover RNA samples from initial diagnosis also ensures robustness to implement the protocol directly in the clinical setting.

Results

Profile of study participants

Early Samples (at first diagnosis) were collected in the first wave of COVID-19 from September 2020 to November 11, 2020, and subsequently in the 2nd wave from March 10 to March 20, 2021. Total 70 COVID-19 early samples, 26 non-SARS-CoV-2 but Coronavirus positive (common cold) samples in total 6 categories (groups 1–6) were recruited as described in Table 1. Additional 16 late samples of ICU admitted COVID-19 patients

Category COVID-19 + /- by RT-PCR		Group (#)		Asymptomatic (A) or symptomatic (S)	Required Oxygen support (Yes /No)	ICU/Death outside ICU (Yes / No)	Comorbidity (Yes/No)	No of samples used		Age (years) (M ± SD)		Sex ratio %M, %F	
Early (first diagnosis) samples of COVID-19 Cases	RdRP + ve E gene + ve	1 * asymptomatic		A	No	No	No	16		37.25 ± 19.25		37%, 63%	
		2 * mild		S	No	No	No	20 (total)	18	47.57 ± 16.32		52%, 48%	
							Yes		2				
		3. Moderate	3a	S	Yes	No	No	9 (total)	4	58.8 ± 14.7	51 ± 18.6	33.3, 66.6%	75%, 25%
			3b	S	Yes	No	Yes		5		65.2 ± 7.1		0%, 100%
		4 Severe		S	Yes	Yes	No	12		42.5 ± 17.9		83%, 17%	
		5. Severe		S	Yes	Yes	Yes	13		51 ± 15.7		38%, 62%	
Early (first diagnosis) samples of common cold	RdRP -ve E gene + ve	6 Non COVID-19 Corona virus + ev		S	No	No	No	15		37.3 ± 11.7		46%, 53.8%	
				A	No	No	No	11		35.1 ± 12.5		36,4%, 63%	
Late stage samples of COVID-19 cases	RdRP + ve E gene + ve	7 ICU admitted at time of sample collection		S	Yes	Yes	NA	16		50.5 ± 18.1		81%, 19%	

Table 1. Study subject groups and distribution of age and sex. *NA* information is not available. *As the groups recover without complication at home quarantine or in general facility we assumed this patient groups should have effective immune response correlated with the good clinical outcome irrespective of present or absent of any comorbidity.

were collected from January to February 2021 (group 7). Age, sex and comorbidity distribution of the samples are given in the Table 1 and Fig. S1. Interestingly, comorbid patients with moderate and severe outcome (groups 3b and 5, respectively) showed female dominance and higher mean age than the corresponding groups without comorbidity (group 3a and 4 respectively).

Average interval from the onset of symptoms to time of sample collection was 4.1 ± 3.2 (0–12) days. No statistical difference was observed in sampling time between mild (group2) and moderate to severe patients (groups 3, 4 and 5). Duration from the date of sample collection to the date of hospital admission for moderate to severe patients (groups 3, 4 and 5) was 0.26 ± 1.34 (1–4) days. For those patients who died due to COVID-19, the mean interval from sample collection to the time of death was 5.1 ± 5.7 (0–18) days (Table S2). Average hospital stay of COVID-19 patients who were treated in ICU and survived was 18.6 ± 9.5 (7–39) days compared to 13.5 ± 4.7(6–20) days for those treated in general facility (Table S3).

Evaluation of T cell response markers for their ability to predict COVID-19 outcome

One of the important adaptive defence mechanisms to control virus infection is T cell activation. We hypothesized that low expression of T cell resting state markers and high expression of activation state markers will represent early virus clearance, indicating a good prognosis.We chose T cell marker genes that represent a resting, naive or suppression state of T cells (T1:FOXP3; T2, ZFP36; T6, CD62L;T7, CCR7) and those indicative of an active state (T3, CD25;T4, CD69;T5, IL17; T8, GZMB). Individual function of the genes is given in Table S3[16,17,30–42].

Initial screening of T cell markers and development of severity score

Initially, we screened the T cell response markers in 10 samples from non-severe group (asymptomatic/mild/moderate groups i.e., groups 1 + 2 + 3) vs. 9 samples from poor outcome groups in which patients either died or survived following ventilator support (i.e., groups 4 + 5). Mean absolute Ct values of all the potential T cell markers (T1—T8) are shown in Table S4. Evaluation of conventional expression of markers (as indicated by β-actin-normalized Δ Ct values) demonstrated that only T2 (ZFP36), T4 (CD69) and T8 (GZMB) had the highest differential expression ($P \leq 0.2$, t-test) between severe and non-severe COVID-19 cases and also had detectable expression in most of the samples (Table 2A, Table S4). While ZFP36 showed lower mean Δ Ct (high expression), CD69 and GZMB showed higher mean Δ Ct (low expression) in severe cases relative to non-severe cases, indicating relatively lower T cell activation in severe COVID-19 cases. Although increased expression of both the genes CD69 and GZMB in non-severe cases relative to the severe cases were detected, only CD69 showed statistically significant increase in expression (0.01). However, differences of individual expression (normalized by β-actin) of the three short-listed transcripts in the two clinical groups were not high enough to be utilized for making a clinical decision. Therefore, a single composite score by rational combination of the individual Δ Ct values was derived to amplify the small difference between the two clinical outcome groups. Thus T2 (ZFP36), T4 (CD69) and T8 (GZMB) were taken for Δ Ct combination analysis for deriving a single composite score as described in Table 2B,C. Interestingly, when Δ Ct values of CD69 and GZMB were derived by subtracting Ct value of ZFP36 (subtraction of Ct of the opposite phase specific markers) instead of β-actin and both the Δ Ct values were combined [ΔT4(T4-T2) + ΔT8(T8-T2)] the resulting score was able to differentiate two clinical groups with the highest level of significance ($P = 0.0006$). On the other hand when the Δ Ct was derived by subtracting two activation phase (same phase) T cell markers (T4-T8), no statistical significance (0.6) was observed between the severe and non-severe groups indicating the robustness of the rational combination method (Table 2C). So the

Principle of Ct value combinations	Non-severe Group (n = 10) Mean ΔCt/ΔCtcombinations	Severe Group (n = 9) Mean ΔCt/ΔCtcombinations	Significance of difference: Severe vs non-severe COVID-19 cases by T-TEST *P*value
A. ΔCt values normalized with ACTB			
T1- ACTB	12.61	13	0.7
T2- ACTB	3.67	3.07	0.20
T3- ACTB	13.87	13.2	0.56
T4- ACTB	**6.47**	**9.49**	0.01*
T5- ACTB	12.38	12.64	0.82
T6- ACTB	6.83	6.34	0.51
T7- ACTB	9.44	9.95	0.67
T8- ACTB	7.89	9.86	0.18
B. Composite ΔCt values by Combination of ACTB normalized ΔCt values			
T4 + T2-2ACTB	10.14	12.56	0.07
T4 + T8-2ACTB	10.70	19.35	0.0027
T2 + T8-2ACTB	11.56	12.93	0.38
C. Composite ΔCt values by combinations of T cell phase specific markers			
T4-T2	2.81	6.42	0.0022
T8-T2	4.22	6.79	0.093
T4-T8	− 1.42	− 0.37	0.60
T4 + T8-2.T2	7.03	13.21	0.0006

Table 2. Deriving best fitted model of 'Severity Score' using the combinations of Ct values of different T cell markers in oro-nasopharyngeal swab RNA. ACTB: β-actin, Ct: cycle threshold of the individual target genes in RT-PCR, ΔCt: Difference of two Ct values as indicated, n: Number of samples, * *P* statistically significance at $P < 0.05$.

Ct value combination score T4 + T8-2T2 was used as 'severity score' for the subsequent sample analysis for its evaluation to predict the disease outcome.

Comparing severity score in the spectrum of COVID-19 outcome

The 'severity score' was distributed normally in COVID-19 and non-COVID-19 control groups as verified by Kolmogorov–Smirnov Test of Normality (Table S5). Mean "severity scores" in different COVID-19 outcome groups have been described in Table 3. Intraday (4–17%) and inter-day coefficient of variation (CV) values were determined (12–21%) and shown in Table S6. We observed a graded increase in severity score along the spectrum of COVID-19 outcome from asymptomatic/mild to death (Table 3). As groups 1 and 2 showed no significant difference in severity score and clinically recovered without any complications, they were combined (7.44 ± 3.26) and used as reference to compare with relatively more severe outcomes. Though group 3 had slightly higher severity score, it was not statistically significant. However, groups 4 and 5 showed significantly higher severity scores (13.86 ± 3.38, $P = 2.2 \times 10^{-5}$ and 12.56 ± 5.71, $P = 7.9 \times 10^{-3}$, respectively). Expectedly, group 5 which consist of severe/critical comorbid patients, showed slightly lower mean value than group 4 which consists of severe/critical COVID-19 patients without comorbidity. Mean severity scores in both the subgroups of samples of group 4 subjects who either succumbed to death or survived following treatment in ICU were higher than in group 5. We also compared these scores with those from symptomatic non COVID-19 corona virus infection (group 6). Interestingly, we observed slightly higher score for symptomatic non COVID-19 corona virus infection but the difference compared to combined groups 1 and 2 was not significant (8.86 ± 3.36, $P = 0.119$).

To understand the diagnostic efficiency of 'severity score', area under ROC curve (AUC) was determined (Fig. 1B). AUC indicated well separation of 'severity score' to identify severe groups from the corresponding non-severe groups. Expectedly, the highest separation was with non comorbid severe group (group 4) (AUC = 0.88, 95% CI 0.73–1) while inclusion of comorbid groups (4 + 5) resulted in slightly less (AUC = 0.82, 95% CI 0.69–0.96) separation from the corresponding non-severe group (1 + 2 + 3) (Figs. 1B, IV and III, respectively). However, combined non-severe groups did not show any separation with group 6 (AUC: 0.61 (95% CI 0.39–0.83) (Fig. 1B, II). Optimum cut off point was determined by the 'cut point to maximize Youden's J and test efficiency'[29]. Interestingly, when we used noncomorbid samples or all the subjects (including comorbid), in both cases the optimum cut off was 12.06 (Fig. 1 BV–VI). This suggests that comorbidity does not affect the baseline value of the score. For practical purpose, we chose a cut off of 12 for the severity score. Therefore, a score more than 12 was considered to be high. Agreement of repeated independent tests in reference to severity score cut off (high/low) has been given in Table S7. Distribution of the severity scores of all the groups (1–6) along the cut off point has been shown in the dot plot[29] in the Fig. 1C.

We observed that out of 25 combined asymptomatic and mild groups (1 + 2), only two samples showed high severity score (8%). On the other hand, 91% ($P = 0.0002$) and 64% ($P = 0.009$) of patients in groups 4 and 5 showed high severity score, respectively. When group 4 (no comorbidity) was divided into dead and ICU (survived) groups, we observed that all 7 patients who died had high severity score (100%, $P = 0.002$) whereas 3 out of 4

Group		Description		N		Severity score (M ± SD) (range)		One sided Significance *P*	%Severity score > 12%(n/N)	Odd ratio OD (95%CI) *P*
1		Asymptomatic		13		7.43 ± 3.15		P = 0.49	7.6 (1/13)	1.09 (0.06–19.6) *P* = 0.9
2		Mild symptomatic		12		7.46 ± 3.51			8.3 (1/12)	
1 + 2		Asymptomatic/mild symptomatic		25		7.44 ± 3.26		Reference	8 (2/25)	Reference
3 (moderate)	3a	O_2 required (HQ/G)	No comorbidity	8	3	8.09 ± 2.44	8.2 ± 1.5	0.28	(0/8)	1.64 (0.13–20) *P* = 0.702
	3b		comorbidity		5		7.98 ± 3			
4 (Severe/critical, No comorbidity)		Death/ICU(Include death outside ICU)		11		13.86 ± 3.38		0.000022	91 (10/11)	115 (9.3–1419) *P* = 0.0002
		Death		7		15.14 ± 2.77		0.00000011	100 (7/7)	141 (6. 1- 3276) 0.002
		ICU (Survived)		4		11.62 ± 4.57		0.08	75 (3/4)	34 (2.4–505.7) *P* = 0.0097
5 (Severe/critical, with comorbidity)		Death/ICU(Include death outside ICU)		11		12.56 ± 5.71		0.0079	64 (7/11)	20 (3.0–134.) *P* = 0.009
		Death		7		14.54 ± 5.73		0.0093	71 (5/7)	28 (3.2–256) *P* = 0.0026
		ICU		4		9.49 ± 4.87		0.23	50 (2/4)	1.5 (1.0–131) *P* = 0.05
4 + 5 (Combined)		Death/ICU(Include death outside ICU)		22		13.21 ± 4.63		0.000010	77 (17/22)	39 (6.7–226) ***P* < 0.0001**
		Death/ventilator required		16		14.54 ± 3.88		0.00000076	88 (14/16)	80.5 (10–638) p < 0.0001
		Death (all)		14		14.72 ± 4.10		0.0000047	86 (12/14)	69 (8.6–552) *P* = 0.0001
		ICU (survived)		8		10.56 ± 4.52		0.051	62.5 (5/8)	19 (2.5–146) *P* = 0.0044
6		Non COVID-19 corona infection		11		8.86 ± 3.36		0.119		1.15 (/0.09–14) *P* = 0.91

Table 3. Association of clinical outcome of COVID-19 with T cell based 'Severity Score' detected in leftover RNA samples of oro-nasopharyngeal swab following COVID-19 diagnosis. HQ:Recovered in home quarantine,G: Patientsrecovered in general hospital facility, N: Number of a particular category, n: number of subjects having severity score > 12 (high risk category) among N, * *P* statistically significance at $P < 0.05$.

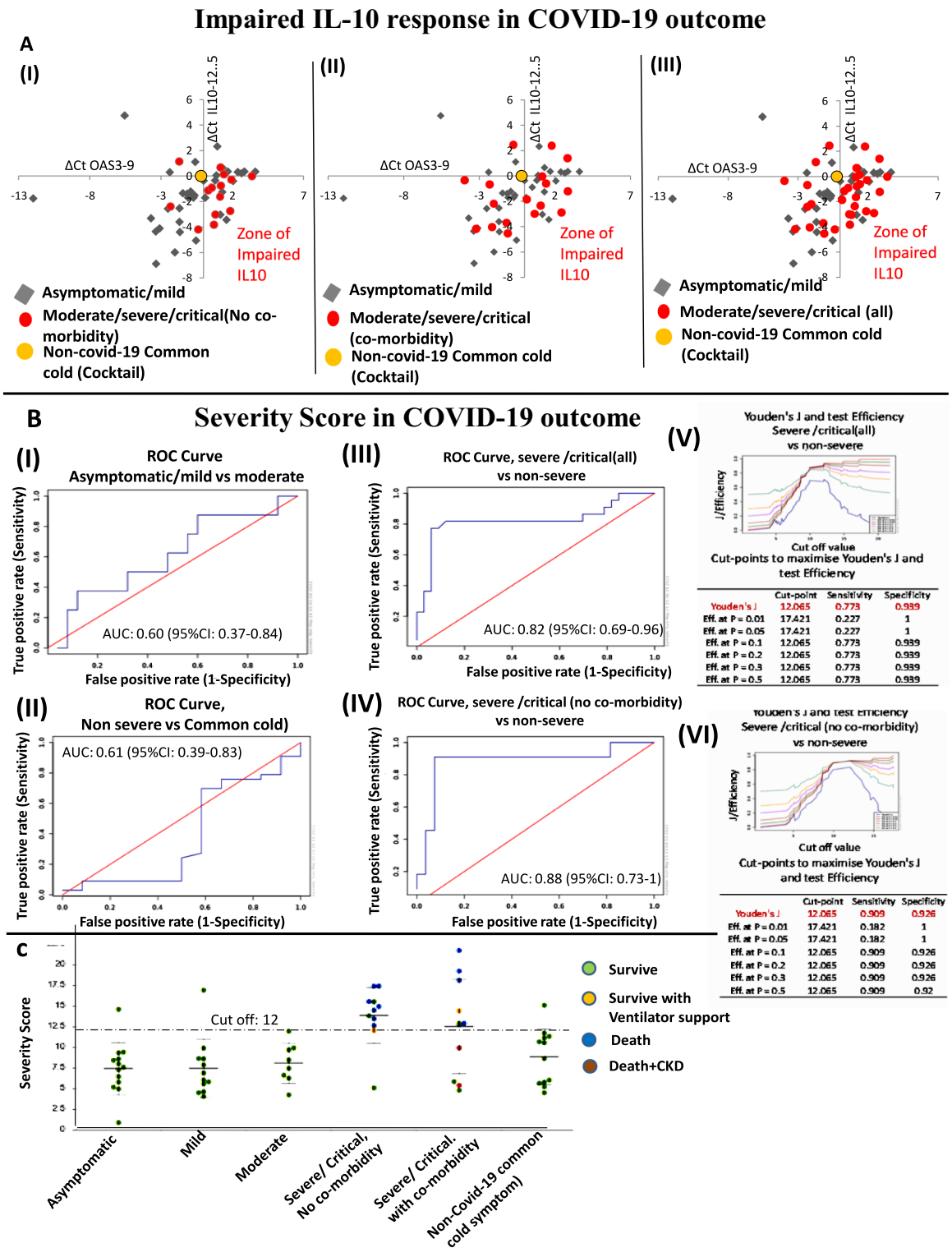
patients who survived had high severity score (75%; $P = 0.0097$). The same in group 5 were 71% ($P = 0.0026$) and 50% ($P = 0.05$), respectively. Interestingly, the two out of seven subjects who died due to COVID-19 in group 5 showed severity score below the cut off value and both the patients had pre-existing chronic kidney disease (CKD). When we combined patients who died and those who survived but required ventilation support, irrespective of the presence or absence of comorbidity, the 'severity score' was 88% (14/16; $P = 0.0001$). None of the 8 samples in group 3 showed high severity score.

For the COVID-19 subjects without comorbidity, sensitivity of detecting severe/critical cases without comorbidity (group 4) was 91% (10/11), (95% CI: 58.7 -99.8), whereas the specificity of 'severity score' for detecting favourable outcome (i.e., with asymptomatic/mild/moderate symptoms) was 92.6% (25/27; 95% CI: 75.7–99). However, combining comorbid and non-comorbid subjects, the sensitivity dropped to 77.3% (95% CI: 54.6–92.2) while specificity slightly increased to 93.9% (95% CI: 79.8–99.3) (Table 4).

We did not find any significant correlation between severity score and duration of illness before the swab sampling among the spectrum of COVID-19 outcome groups (Table S7a). However in milder outcome group (group 2) a weak non-significant negative correlation (R = − 0.24) while in severe group (group 4 + 5) a weak non-significant positive correlation (R = 0.31) with duration of illness before the swab sampling for the test were observed (Table S7a). Severity score was moderately correlated with duration of hospital stay (R = 0.55, P = 0.04, Table S7b). We also did not find any association of the 'severity score' with age and sex (Table S8) suggesting that these variables have little effect on the observed difference in severity scores among different COVID-19 outcome groups.

Profile of comorbidities found in this study is described in Table 5. Diabetes mellitus (DM) was the most frequent comorbidity which was 50% (9/18) of the total comorbid patients including mild, moderate to severe COVID-19 outcome groups (group 2, 3, 5). Out of 9 DM patients, cases having DM alone, combination of DM with hypertension (HTN) and combination of DM with chronic kidney disease (CKD) were 5, 3 and 1 cases respectively. Next frequent comorbidity was HTN (7/18) which was either alone (4 cases) or in combination with DM (3 cases). Interestingly, when DM was the only comorbidity, all the 5 DM patients had low severity score (< 12) and were survived even though one of the patients had insulin dependent DM**.** However, out of 4 HTN patients (alone), 2 had higher severity score while 1 succumbed to death. In combined DM + HTN, out of 3 cases 2 had higher severity score while 1 succumbed to death. Rest of the comorbidity including cancer, anaemia, TB and all the affected subjects had high severity score and succumbed to death.

To understand if T cell response as represented by 'severity score' may synchronize/associated with antibody response to SARS-CoV-2 infection, 16 paired samples of oro-nasopharyngeal swab VTM samples and blood



samples were collected from COVID-19 patients who were already admitted to ICU (group 7, Table 1). So, we consider this samples as representative of late response of immune system to COVID-19. We could not perform this experiment with early samples because at the time of sampling research team was not in contact with subjects and the recruitment was done retrospectively following outcome of the disease. We also could not detect anti-SARS-CoV-2 Spike protein antibody in the collected swab samples in VTM. Following RNA analysis of the swab samples (group 7), 6 samples were identified as having low 'severity score' while 10 samples were identified as having high severity score. We observed low severity score group have little lower mean anti-spike protein plasma antibody than high severity group without any statistical significance (552.87 ± 413.19 and 1622.74 ± 1816.36 respectively, P = 0.10). We also observed very weak positive corelation (R = 0.29, P = 0.23) between severity score and plasma anti-spike protein antibody level in the ICU admitted patients (Table S7c).

◂**Figure 1.** 'Impaired IL-10 response' and 'Severity score' analysis in different COVID-19 outcome groups. (**A**) (ΔCt IL-10–12.5) and (ΔCt OAS3-9) were plotted in X and Y axis respectively. If the (x,y) quadrant of a representing a sample, fall on the lower right quadrant of the plot area will have ΔCt IL-10 > 12.5 and ΔCt OAS3 < 9. This area is considered as zone of "impaired IL 10". After plotting if a sample values fall on this area the sample is considered have "impaired IL-10". Distribution of "impaired IL-10 response" in non comorbid moderate/severe (1), comorbid moderate/severe (II) and combined moderate/severe COVID-19 outcome groups relative to asymptomatic/mild groups. The points indicating different groups are indicated in the figure with respective colour code. (**B**) Receiver operator characteristics (ROC) curves of 'Severity Score' for evaluating its performance as prognostic test for identifying the clinical outcome groups which may give best separation from the corresponding control groups. (I) ROC curve for evaluating separation of moderate COVID-19 group (group 3) from asymptomatic/mild group (group 1 + 2), (II) ROC curve for evaluating separation of Asymptomatic/mild/moderate (group 1 + 2 + 3) COVID-19 group from non COVID-19 corona virus infection(common cold) (group6), (III) ROC curve for evaluating separation of all severe/critical COVID-19 group (group 4 + 5) from non-severe groups (group 1 + 2 + 3), (IV) ROC curve for evaluating separation of severe/critical COVID-19 group without comorbidity (group 4) from non-severe groups without comorbidity (group 1 + 2 + 3a). (V) & (VI): Determining Cut-points to maximize Youden's J and test Efficiency. (V) Cut off for prediction of all severe/critical COVID-19 group (group 4 + 5) from non-severe groups (group 1 + 2 + 3), (VI): Cut off for prediction of severe/critical COVID-19 group without comorbidity (group 4) from non-severe groups without comorbidity (group 1 + 2 + 3a). AUC: Area under ROC curve; CI: Confidence Interval. Red diagonal line indicates true positive rate (Sensitivity) and false positive rate (1-Specificity) are equal (AUC:0.5) suggesting no prognostic value. (**C**) Dot plot showing distribution of Severity Score in the spectrum of COVID-19 outcome. Black dashed line indicate cut off point of the Severity Score determined by Cut-points to maximise Youden's J and test Efficiency. Asymptomatic: Group1 cases, Mild: Mild symptomatic without requirement of O_2, Moderate: cases required O_2 but treated in general facility or at home (Group 3), Severe/critical with no comorbidity: treated in ICU or death due to COVID-19 irrespective of treatment and having no comorbidity (group 4), Severe/critical with comorbidity: treated in ICU or death due to COVID-19 irrespective of treatment and having comorbidity (group 5), Non-COVID-19 common cold symptom: symptomatic non-COVID-19 corona virus positive as diagnosed by corona virus E gene positive by RT PCR (Group 6). Green circle: patients who survived, blue circle: patients who died due to COVID-19, yellow circle: patients who survived with ventilator support, brown circle: patients who died due with COVID-19 and having chronic kidney disease (CKD).

Evaluation of innate immune response gene expression in oro-nasopharyngeal swabs

We first screened the leftover RNA samples for expression of a representative interferon response gene (OAS3), an inflammation marker (IL-6) and an anti-inflammatory response marker (IL-10). The target gene expression was normalized by housekeeping gene β-actin. When we compared overall normalized mean ΔCt values of all three genes in asymptomatic/mild groups (groups 1 + 2) and moderate/severe groups (groups 3 + 4 + 5), only IL-10 showed slightly higher ΔCt (1 cycle difference) value in moderate/severe groups (groups 3 + 4 + 5) whereas no deference was observed in ΔCt values of OAS3 and IL-6 (Table 6)mean Ct value of β-actin was comparable in both the asymptomatic/mild and moderate/severe groups. Percentage of samples with no detectable IL-10 expression (as per our criteria ΔCt > 12.5) was also slightly higher in severe infection group compared to milder group (64% vs. 44.4%, P = 0.1) without statistical significance. OAS3 showed 100% detectable expression in all the samples (Table 6). The percentage of patients with no detectable IL-6 expression was also not significantly different in asymptomatic/mild groups and moderate/severe groups (Table 6). Interestingly impaired IL-10 response, as defined by nodetectable IL-10 expression (ΔCt > 12.5) but high OAS3 expression (ΔCt < 9), showed significantly higher proportion in moderate/severe relative to asymptomatic/mild groups ($P = 0.006$) (Table 6).

The proportion of 'impaired IL-10 response' in different COVID-19 outcome subgroups is described in Table 7. Since the percentage of patients with impaired IL-10 response in asymptomatic group (group 1) was not different from that in mild group (group2), the two groups were combined and used as reference for comparison with more severe outcome groups. Among group 4 and 5 patients who died, 66.6% and 62.5%, respectively (P ≤ 0.03) had impaired IL-10 response. This data indicates a significant association of impaired IL-10 response with poor clinical outcome. The association of impaired IL-10 response with poor clinical outcome was statistically stronger in combined critical groups (death or survived with support of ventilator, irrespective of comorbidity) (64.7%, P = 0.0038) compared to combined asymptomatic/mild groups. Interestingly, among the comorbid subjects, percentage of patients with impaired IL-10 response in groups with moderate outcome (subgroup 3b, Table 1) and ICU admission (but survived) were 40% and 0%, respectively, whereas in non-comorbid groups, the same were 66.6% and 60% respectively (Table7). In Fig. 1, the distribution of IL-10 response among COVID-19 subjects without and with comorbidity is shown as indicated. It seems that among non-comorbidity subjects, detection of impaired IL-10 response was more consistent across the spectrum of COVID-19 outcome from moderate to critical patients. There was slightly higher frequency of impaired IL-10 response (27.2%; 3/11) in non-COVID-19 Coronavirus infection (common cold, 11 samples) compared to asymptomatic/mild COVID-19 group (19%), however the difference was not statistically significant. We also tested 'impaired IL-10 response' in three severe swine flu samples which were qualified for analysis as per our criteria set for COVID-19. Out of the three swine flu samples of hospital admitted patients, two were detected as 'impaired IL10 response (66%).

No statistically significant association of 'impaired IL-10 response' with age and sex was observed in asymptomatic/mild (1 + 2) or moderate/severe (3 + 4 + 5) groups (Table S9).

Among non-comorbid COVID-19 subjects, impaired IL-10 response was able to detect moderate to severe COVID-19 outcome (groups 3a and 4) as a combined group with an sensitivity 64.2% (95% CI 35.1–87.2) and

Statistics		Performance of 'Severity Score'				Performance of 'impaired IL-10 response'			
		COVID-19 outcome: Requiring ICU support /death (No comorbidity)		COVID-19 outcome: Requiring ICU support /death (with or without comorbidity)		COVID-19 outcome: Requiring O2/ICU support /Death, No comorbidity		COVID-19 outcome: Requiring O_2/ICU support /death (with or without comorbidity)	
		Yes (N)	No (N)	Yes (N)	No (N)	Yes (N)	No (N)	Yes (N)	No (N)
Confusion Matrix	Risk Detected	10	2	17	2	9	6	16	7
	Risk not detected	1	25	5	31	5	28	15	29
	Total	11	27	22	33	14	34	31	36
Test performance		% (95% CI)		% (95% CI)		% (95% CI)		% (95% CI)	
Sensitivity		91 (58.7 -99.8)		77.3 (54.6–92.2)		64.2 (35.1–87.2)		51.6 (33.1–69.8)	
Specificity		92.6 (75.7–99)		93.9 (79.8–99.3)		82 (65.5–93.2)		80.5 (64.0–91.8)	

Table 4. Performance of 'severity score' and 'Impaired IL-10 response' as diagnostic test identifying outcome of COVID-19. Risk detected: Severity score > 12 or 'Impaired IL-10 response' detected; Risk not detected: Severity score < 12 or 'Impaired IL-10 response' not detected; N: Number of cases, CI: Confidence Interval.

Comorbidity	No of cases	High severity score (> 12)	COVID-19 Outcome	
			Death	Survived
DM	5	0	0	5
DM + HTN	3	2	1	2
DM + CKD	1	0	1	0
CKD	1	0	1	0
HTN	4	2	1	3
Cancer	1	1	1	0
Anaemia	1	1	1	0
TB	1	1	1	0
Thyroid	1	0	0	1
Total	18	7	7	11

Table 5. Comorbidity profile along with severity score and COVID-19 outcome. DM: Diabetes mellitus, HTN: Hypertension, CKD: Chronic kidney disease, TB: Tuberculosis.

Mode of analysis	Potential biomarkers of innate immunity response	Group 1 + 2(36) Mean ± SD	Group 4 + 5 + 3(31) Mean ± SD	T-TEST (*P*)	Cocktail of 15 sample of group 6S
ΔCt values normalized with ACTB	IL-10-ACTB	11.77 ± 2.8	12.75 ± 1.9	0.1	12.3 ± 0.4
	OAS3-ACTB	7.35 ± 2.35	7.57 ± 1.9	0.7	9 ± 0.6
	IL6-ACTB	11 ± 3.2	11 ± 2.8	1.0	12.2 ± 0.75
		% No expression/ ΔCt > 12.5	% No expression/ΔCt > 12.5		
% no expression or ΔCt > 12.5	IL10-ACTIN	44.4% (16/36)	64.5% (20/31)	0.1	
	OAS3-ACTIN	0%(0/36)	0%(0/31)	1.0	
	IL6-ACTIN	42%(15/36)	45% (14/31)	0.80	
Composite model: low IL10 in the background of high OAS3 expression	ΔCt Il10 > 12.5, ΔCt OAS3 < 9	19.4% (7/36)	51.6% (16/31)	0.006*	

Table 6. Comparison of normalized Ct values of IL-10, OAS3 and IL-6, in assymptomatic/mild and moderate/severe groups and modelling for detecting severe group. ACTB: β-actin ,ΔCt : Ct difference between target gene—β-actin,* *P* statistically significance at $P < 0.05$.

specificity 82% (95% CI 65.5–93.2). However, combining comorbidity reduced the sensitivity to 52% (95% CI 33.1–69.8), whereas specificity was still 82.8% (95% CI 64.0–91.8) (Table 4).

Undercurrent principle of Severity Score and impaired IL-10 response in determining COVID-19 outcome have been shown in the schematic diagram in Fig. 2.

Group		Description		N		Impaired IL-10 expression: % (n/N)		Fisher exact probability *P*		Odd ratio OD (95%CI) *P*	
1		Asymptomatic/		16		18.7 (3/16)		1.0 (group 1 vs 2)		1 (0.2–5.7) p = 0.92 (group 1 vs 2)	
2		Mild		20		20 (4/20)					
1 + 2		Asymptomatic/mild		36		19 (7/36)		Reference		Reference	
3 (moderate)	3a	O_2 required (HQ/G)	No Comorbidity	8	3	50 (4/8)	66.6 (2/3)	0.09	0.13	4 (0.8–20.7) *P* = 0.084	-
	3b		Comorbidity		5		40.0 (2/5)		0.3		-
4 (Severe/critical, No comorbidity)		Death/ICU (Include death outside ICU)		11		64 (7/11)		0.009*		7.25 (0.72–12) *P* = 0.0087*	
		Death		6		66.6 (4/6)		0.03*		8.2 (1.25–54) *P* = 0.028*	
		ICU (survived)		5		60 (3/5)		0.08		6.2 (0.87–44) *P* = 0.069	
5 (Severe/critical, with comorbidity)		Death/ICU (Include death outside ICU)		12		41.7 (5/12)		0.14		2.9 (0.71–12) *P* = 0.13	
		Death		8		62.5 (5/8)		0.023*		6.9 (1.3–36) *P* = 0.02*	
		ICU(survived)		4		0 (0/4)		1		0.437 (0.02–9) *P* = 0.59	
4 + 5		Death/ICU (Include death outside ICU)		23		52 (12/23)		0.01*		4.4 (1.3–13) *P* < 0.0177*	
		Death/ventilator required		17		64.7 (11/17)		0.0038*		6.9 (1.87–25) *P* = 0.0037*	
		Death (all)		14		64 (9/14)		0.005*		6.6 (1.65–26.6) *P* = 0.0076*	
		ICU (survived)		9		33 (3/9)		0.39		2 (0.4–10) *P* = 0.37	
6		Non COVID-19 Corona virus + ev(common cold)		11		27.2% (3/11)		0.68			

Table 7. Association of clinical outcome of COVID-19 with 'impaired IL-10 response' detected in leftover RNA samples of oro-nasopharyngeal swab following COVID-19 diagnosis. Impaired IL-10 expression: defined as ΔCt IL-10 > 12.5, ΔCt OAS3 < 9 in RT PCR (low/undetectable IL-10 expression on the background of high pro inflammatory OAS3 expression), HQ: Recobered in home quarantine, G:Patients recovered in general hospital facility, N: Number of particular category, n: number of subjects having impaired IL-10 expression (high risk category) among N. *P statistically significance at $P < 0.05$.

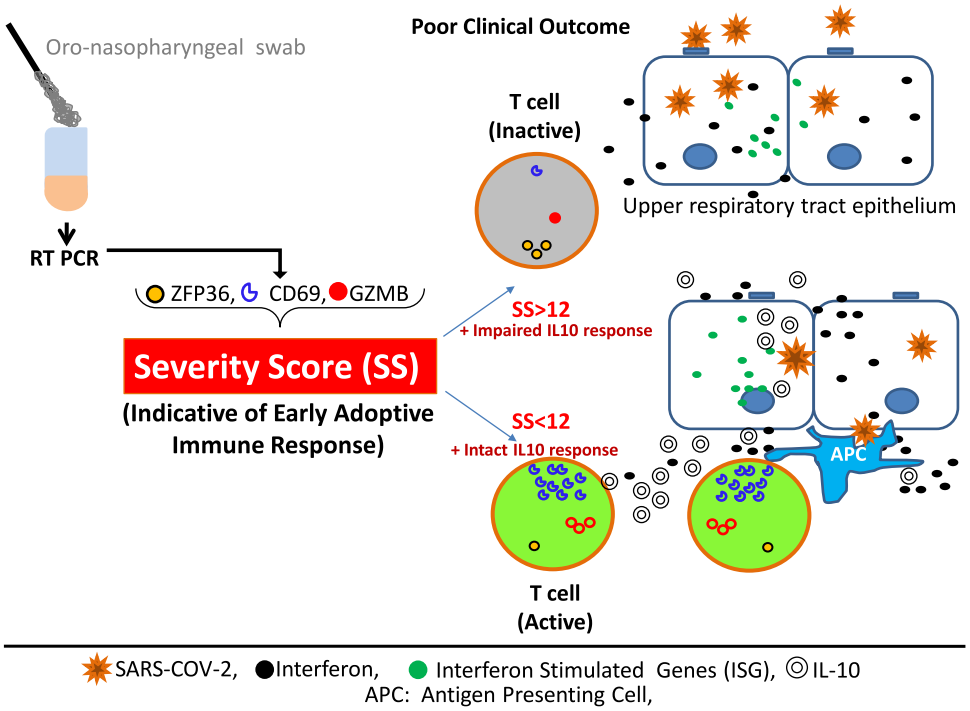


Figure 2. Schematic diagram showing under current principle of Severity Score and impaired IL-10 response in determining COVID-19 poor outcome.

Discussion

In this study, we investigated if leftover RNA samples of oro-nasopharyngeal swabs following SARS-CoV-2 diagnosis can be used to evaluate potential of T cell responses and impaired IL-10 as early prognostic biomarker/s for segregating the high risk COVID-19 group. We have taken two different approaches for evaluating these two different classes of biomarkers. Considering the complexity and inconsistency expected in detecting gene expression in mixed cell populations of oro-nasopharyngeal swabs, we aimed to develop a comprehensive strategy to make a robust detection system which could reduce or nullify the effect of technical variabilities. The conventional approach of host gene expression analysis of an individual marker in a complex tissue, normalized by a house keeping gene, has very little interpretable value and its results are rarely reproducible to be used for clinical decision making for individual subjects. Here, we used rational combination of genes based on their specific biological roles and the results provide much robust, stable and predictable clinical outcome.

CD69 is one of the early T cell activation markers[36,37] while GZMB gene product granzyme B is expressed in matured T cells inducing a killer phenotype[43]. ZFP36 is a newly characterized master regulator of T cell activation controlling post transcriptional regulation. The gene keeps T cell in inactive stage by its expression of ZFP RBP protein in resting phase and its down regulation upon acute viral infection bring T cell to an activated state[33]. Expectedly, relatively low expression of ZFP36 was also consistent with high expression of CD69 and moderately high expression of GZMB in non-severe outcome group of COVID-19 compared to severe outcome group. Together these markers indicate a state of early T cell activation (Table 2A). Moderately high expression of GZMB might indicate that a small fraction of T cells is in a state of maturity to killer phenotype at the time of sample collection contributing to a favourable outcome.

In previous reports, it was shown that acute respiratory distress syndrome (ARDS) appears between 8–9 days of symptomatic COVID-19 infection[1,44]. Our average sampling timing was at 4.1 ± 3.2 days of illness, which is earlier than initiation of inflammatory dysregulation and ARDS. This sampling timing also matched with the reported earliest detection of T cell response against SARS-CoV-2 infection by ex vivo experiment, i.e., within 4 days from illness, whereas detection of circulating activated T cell has been reported to be at day 7 of infection[27,28,45]. Local T cell activation to SARS-CoV-2 infection in upper respiratory tract may take place earlier than its appearance in circulation. Presence of SARS-CoV-2 specific T cells is associated with less severe disease[46–48]. As dysregulated inflammation is initiated when initial immune response fails to control virus infection, it is feasible to use the T cell activation marker in early stage of infection (prior to activation of inflammatory markers) for predicting disease outcome and our results establish the proof of the principal in this regard.

Previously, it was demonstrated that failure of early T cell response followed by high antibody production was associated with more severe form of COVID-19[27,46,47]. We have also observed slightly an increased plasma anti-spike protein antibody level in high severity score patients (low T cell activation) compared to low 'severity score' ICU admitted COVID-19 patients. However, we are cautious to conclude this because the difference was not statistically significant. The increased antibody production in response to low T cell response might be due to a compensatory immune mechanism. However, it has been reported that the antibody response is not likely to be correlated with the viral clearance[47–49] or the outcome of the disease.

For evaluating impaired innate immunity, initially we observed no statistically significant deference in β-actin normalized IL-10 and OAS3 expression in moderate/severe cases relative to asymptomatic/mild cases of COVID-19. As IL-10 response increases along with increasing immune (innate or adaptive) response for maintaining immune homeostasis[23,50–52], mere increase or decrease of IL-10 can't accurately reflect dysregulation of innate immune response. There should be lack of IL-10 response in the background of high innate immune or pro inflammatory response. Therefore, we defined 'impaired IL-10 response' as low expression of IL-10 (indicated by high ΔCt) accompanied by high expression of OAS3 representing high expression of ISGs (indicated by low ΔCt of OAS3). This model resulted in high significant level of difference when we compared the two clinical outcome groups of COVID-19.

Though dysregulated inflammation or cytokine storm plays a detrimental role in outcome of COVID-19, we did not observe increased expression of IL-6 in moderate/severe group compared to asymptomatic/mild groups. This seems to be in contrast to many previous study which correlated IL-6 up-regulation in blood with deregulated inflammation and COVID-19 severity[53], however early local immune response and cell types involved in oro-nasopharyngeal mucosa are different than systemic late response and cell types in blood. Our result suggests that there might not be difference in pro-inflammatory signalling in early stages locally in asymptomatic/mild or severe COVID-19 cases at least in upper respiratory tract epithelium. However, local impaired IL-10 response may fail to check pro-inflammatory signalling in a timely manner leading to uncontrolled disseminated inflammatory reaction in later stage which is yet to be verified in future study. The observation that association of local (at the site of entry) 'impaired IL-10 response' with severe/critical COVID-19 outcome, is also well aligned with a previous observation which showed that locally induced IL-10 could limit cutaneous vaccinia virus spread[24]. In our study, the reason that we did not detect IL-6 upregulation in severe compared to mild cases, suggests that timing of our sample collection was prior to emergence of dysregulated inflammation at least in upper respiratory tract. Detection of the highest percentage of no detectable expression of IL17A (a marker for Th17 T cells responsible for inflammatory tissue injury[34,35] in severe and non-severe COVID-19 outcome groups (Table S4) additionally supports the observation. Interestingly, Th17 differentiation is also induced by IL-6[54].

Production of IL-10 following acute virus infection coincides with the activation of adaptive antivirus T cell response[50–52]. So, IL-10 response seems to be detectable earlier i.e., before dysregulated inflammatory phase or cytokine storm in COVID-19 illness. This is consistent with our finding demonstrating impaired IL-10 and T cell responses but no change in IL-6 locally at the time of diagnosis in severe COVID-19 outcome groups relative to mild and asymptomatic groups. Interestingly, our results also showed T cell response to asymptomatic/mild/moderate COVID-19 cases dose not differ from response to common cold by less virulent non-COVID-19

corona virus infection suggesting that the T cell 'severity score' could predict T cell response irrespective of the virus strain.

Comorbidity may play an independent role in modulating the COVID-19 outcome. This might be a reason for low sensitivity of both the approaches (impaired IL-10 response and T cell based severity score) to detect potential severe comorbid COVID-19 cases. However, impaired IL-10 response was less effective to predict high risk comorbid COVID-19. If comorbidity affects through impairment of adaptive immune response, the high-risk prediction will be more sensitive to be detected by the severity score. However, interestingly the proportion of the severity score to detect noncomorbid COVID-19 patients who died was 100%, whereas in comorbid group the score only failed to detect two patients who had CKD as high risk factor. CKD seems to act as an independent high risk factor in fatal COVID-19 outcome[55]. It is possible that the presence of CKD adversely impacts COVID-19 patients independent of T cell activation status. If true, this could be the reason that both CKD patients died even though severity score was low. However, other comorbidities such as TB, anaemia, hypertension, cancer and diabetes in the critical patient group do not seem to affect the risk assessment by our severity score. This suggests that if these chronic diseases (other than CKD) have any role in COVID-19 patient outcome, it might be through their impact on immune system and T cell response against SARS-CoV-2. However, very low sensitivity of 'impaired IL-10 response in segregating moderate/severe COVID-19 among comorbidity groups might be related to pre-existing general inflammatory conditions associated with these chronic diseases[56–60]. Interestingly, previously discovered inflammatory biomarker "calprotectin" also showed variable upregulation in different chronic diseases and lifestyle exposures[61,62] suggesting low specificity as well as need for adjusting baseline values to predict severity in comorbid COVID-19 patients. However, in our study, severity score in comorbid mild or moderate cases remained lower than the cut-off value just like no comorbid cases of the same groups (group 1, 2 and 3). Moreover, average score in groups 5 and 4 were comparable (Table-3) and was not statistically different. Additionally, cut-off values derived by Youden's J and for both with or without comorbid subjects were similar. Together, the observations suggest that presence of comorbidity does not affect any change of base line value of the severity score increasing the specificity and reliability of the test in a spectrum of patient profile unlike inflammation based biomarkers.

There might be difference in relative ratio of pro and anti inflammatory response to different infections for an eventless recovery. This might be the reason that we observed slightly higher (although statistically non-significant) frequency of 'impaired IL-10 response' to non COVID-19 corona virus infection (common cold) compared to mild COVID-19 group. Though very limited severe swine flu samples were tested at the time of submission, however consistent detection of high proportion 'impaired IL-10 response' indicate universal applicability of the method to detect clinical outcome. However separate larger study for each respiratory tract infection will be required for establishing its applicability and setting cut off value.

Although non-significant, the correlation of severity score with duration of illness (from disease onset to time of sampling) was negative in the good outcome group whereas positive in the poor outcome group. Although remains to be confirmed in a larger longitudinal study, the negative correlation may reflect progressive activation of T cell response contributing to good outcome whereas positive correlation indicates worsening of T cell response with time. The observation also supports the previous report that fatal COVID-19 outcome is associated with lack of T cell response even after 10 days of infection[26,27].

Though 'severity score' is able to predict potential extreme severe cases with high sensitivity and specificity, it cannot distinguish moderate COVID-19 patients who may require oxygen support in general facility (group 3) from mild symptomatic or asymptomatic groups. On the other hand, impaired IL-10 response-based test could not differentiate between group 3 and severe groups (4/5). If we take the opportunity to combine both the tests (T cell based 'severity score' and impaired IL-10 response) in a sequential manner, the patients with low severity score (< 12) and impaired IL-10 response (excluding comorbidity cases) it could be possible to segregate cases having risk of oxygen requirement without requirement of ICU facility. Though test of impaired IL-10 response may have low sensitivity and specificity, however the additional test on low-risk severity score samples will give an opportunity to detect any high-risk sample which might have been missed by the first test, along with moderately risk samples. Keeping this category at oxygen supported facility before time would be life saving during oxygen crisis-associated COVID-19 waives. In a follow-up study, it will be needed to evaluate spectrum of comorbidities which may or may not have any impact on risk assessment by the severity score.'Individual Case Examples' 1–3 are presented in the supplementary document as model examples (Tables S10-S14), for its potential use in clinics.

As the hyper inflammation is final outcome of impaired immune response to SARS-CoV-2, identification of a new inflammatory biomarker may have little benefit for application in clinical management. However, unlike previous studies, our test will not only identify potentially critical COVID-19 cases for early management, but also provide an etiological insight in immune response impairment which allows an opportunity for additional therapeutic exploration before the condition gets worse. This is the first demonstration of a comprehensive model of using biomarkers identified in leftover swab samples for management of COVID-19. Further study may explore if controlled T cell modulators may overcome the impairment and provide a favourable outcome of COVID-19. Additionally, as most of the anti-viral drugs are toxic, expensive and most effective when administrated in early phase of the infection, applying those drugs to the targeted high risk patients at early phase of the disease based on 'severity score' would be a significant improvement in COVID-19 treatment policy as well as health care management.

Although a few biomarker studies have been reported previously using oral swab samples, none has detailed description on how it could be applied in the field considering the lack of uniformity in high quality sampling. However, in this work we have described a comprehensive SOP considering all the possible field conditions and could also be useful for establishing other swab-based biomarkers. Though sample size was small, robustness of our methodology is able to detect critical COVID-19 cases from mild/asymptomatic groups with a high statistical significance. As per our knowledge, this is the first report showing a novel metric based approach of scoring

early adoptive immune response which could be used as predictor of severe COVID-19 outcome at the time of first diagnosis using the same leftover swab RNA. This work is also a first translational application of the newly discovered role of ZFP36[32] along with CD69 and or GZMB as indicator of T cell response. In future study, it will be important to evaluate 'severity score' in post vaccinated break through infection of SARS-CoV-2. As the 'severity score' represents common T cell response to a viral infection, it has potential to be applied to any other viral infection also. However, as the background T cell immunity and immune reactivity to an infection may vary in diverse populations, the cut off value for 'severity score' may need to be re-set for its applicability for different infections in different populations.

Limitation of the study

It is a pilot study establishing the proof of the concept. The proposed outcome is evaluated in a small number of samples. In this study, we collected clinical information from recovered patients through direct communication with them (over the phone) or with their relatives if the patients died. Hospital data was also used wherever available. So, we included only basic clinical parameters which could be retrieved and verified in most of the patients for categorizing them. As the COVID-19 illness starts very vaguely, most of the patients could not recall precisely the time of onset of the illness, therefore, actual duration of illness before the diagnosis of COVID-19 could differ. As the study design demands retrospective sample shorting following prospective outcome, it was not possible to carry out other conventional blood based markers of severity for correlation with the study markers in upper respiratory tract.

Materials and methods

Ethical statement

Study was carried out at Multi-Disciplinary Research Unit (MRU), Pt. Jawaharlal Nehru Memorial Medical College, Raipur Chhattisgarh, India and Dr. B.R.A.M. Hospital Raipur, C.G., which was a designated COVID-19 hospital (under Pt. Jawaharlal Nehru Memorial Medical College, Raipur) following approval of the protocol by institutional ethical committee. All procedures were carried out in accordance with relevant guidelines and regulations. Human samples were collected following informed consent from the study subjects.

Sample collection strategy and study participants

Considering very low percentage of critical cases (5%) and at least 8–12 days of lag period for developing severe COVID-19 following initial appearance of symptoms, it is very challenging to design a study where initial oro-nasopharyngeal swab samples of severe COVID-19 cases could be collected and analysed. If a study collects samples at the point of first diagnosis, it is only able to report data related to symptomatic or asymptomatic. At the time of diagnosis, most of the patients may not show severity. If it is followed up, only very few subjects may turn into severe/critical outcome of COVID-19. On the other hand, if study starts following the outcome of the disease, it is not practically possible to collect retrospectively the early samples from the respective diagnostic laboratories. It is because at the time of pandemic every day huge number of samples is deposited in diagnostic laboratory limiting the storage capacity and it is discarded time to time. It also may not be practical to collect samples retrospectively from different laboratory.

To overcome these problems, unlike conventional collection of samples at the time of hospital admission, we took an innovative approach. The leftover RNA samples as well as leftover oro-nasopharyngeal swab samples in VTM following SARS-CoV-2 diagnosis were collected and stored en-mass blindly from COVID-19 diagnostic laboratory of Pt. J.N.M. Medical College, Raipur, Chhattisgarh, India for a specified period during the first and second wave of COVID-19 pandemic. Primary information was retrieved from the designated COVID-19 portal of the institute for the same time period. Following the lag period for the outcome of the disease (>10 days), interview (over the phone) was taken for screening of the samples for specific category based on the final outcome, and if the patient's profile matched the inclusion/exclusion criteria and the patient agreed to give informed consent (over the phone), the sample was included for the study. In case if the patient died, the consent was taken from the close relative whose phone number was registered during the sample submission and/or patient's admission to the hospital. Additionally, we collected COVID-19 ICU data from B.R.A.M. hospital (which is also under Pt. J.N.M. Medical College, Raipur) for the information of COVID-19 patients admitted during the time period. Swab samples of all the COVID-19 patients admitted in this tertiary care hospital are submitted to the COVID-19 diagnostic laboratory of Pt J.N.M Medical College Raipur, Chhattisgarh, India for SARS-CoV-2 diagnosis by RT-PCR. The ICU patients basic information was matched with the sample information in the COVID-19 portal of Pt. J.N.M. Medical College, Raipur during the period of collection using filtering option in excel file. The matched shortlisted COVID-19 positive subjects were telephonically verified following interview and if matched the informed consent was taken. Subsequently samples were short listed for final RT-PCR analysis. Those samples which were not matched or telephonically verified were excluded from the study. Criteria for oxygen therapy at home or general facility (group 3) and ICU admission (group 4/5) toCOVID-19 patients were followed as per national guideline for COVID-19 management (AIIMS/ICMR-COVID-19 National Task Force/ Joint Monitoring Group (Dte.GHS), Ministry of Health and Family Welfare, Government of India, issued Clinical Guidance for Management of Adult COVID-19 Patients).

RT-PCR reaction for host cell biomarker detection

cDNA preparation

Leftover RNA samples (9 µl each) from oro-nasopharyngeal swabs following SARS-CoV2 diagnosis were used for cDNA preparation using Superscript III first strand synthesis Kit (Thermo scientific, US) as per manufacturer's protocol. As our input material is the leftover RNA sample prepared in the clinical COVID-19 diagnostic

laboratory, we assumed RNA may lose some integrity. So random hexamer was used for preparing cDNA instead oligo dT which binds to poly A region of mRNA. 9 µl RNA, 1 µl of Random Hexamer and 1 µl of dNTP were mixed and incubated at 65 °C for 5 min and then place in ice for cooling. Then 10 µl of cDNA synthesis mix containing RT buffer, $MgCl_2$, DTT, RNase out, SuperScript III RT (200U/ µl) was prepared as per manufacturer protocol and added to the RNA, random hexamer, dNTP mix. Program for cDNA synthesis was as follows: 10 min at 25 °C, 50 min at 50 °C and terminate the reaction at 85 °C for 5 min. For minimizing batch variation same day's severe COVID-19 samples (group 3/4/5) were processed with the milder/asymptomatic (group 1/2) samples of the same day.

qPCR reaction and thermo cycle conditions

All RT-PCR target genes and the corresponding primer sequences used in this study have been given in Table S1. Primers were designed flanking one or more intron site and it was experimentally confirmed that no genomic DNA was amplified. Each qPCR reaction was performed in 20 µl volume containing 2 µl cDNA mixture, 1 µl of each respective primer pair mixture (10 pM of each forward and reverse primer/µl) and 1X Power up SYBER green master mix (Thermo scientific, US). PCR was run on a Bio-Rad RT-PCR instrument (Bio-Rad). PCR programme was as follows: 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s.

RT-PCR analysis

Sample criteria for impaired IL-10 response and 'severity score' analysis

For all the markers analysed, Ct value was considered up to 40 cycles. If no amplification was detected, for uniformity in analysis, a Ct value of 40was used for such samples. As during viral RNA isolation from oral-nasopharyngeal swab carrier RNA is used routinely in the extraction kits to increase the yield, practically there is no scope to normalize the RNA quantity before RT PCR. Rather we used a cut off Ct value of the most abundant transcript as limit of acceptance of the samples. We observed that in the oro-nasopharyngeal RNA samples, IL-10 and IL-6 expression are detected at very late cycle and when Ct value of β-actin was > 27.5 cycle, detectability in both mild and critical groups reduced drastically. So in our criteria for acceptability of samples for innate immunity response evaluation, a cut off Ct value of β-actin was set as 27.5. If Ct of β-actin in any sample showed > 27.5 the sample was considered undetermined and was excluded from analysis. So acceptable limit of detection of ΔCt IL-10, OAS2 and ΔCt IL-6 in samples having β-actin at the cut-off limit, were considered up to 12.5 (40–27.5). So for a practical purpose any ΔCtvalue > 12.5 was considered as no detectable expression. For T cell analysis as T2 (ZFP36) showed highly expressed gene in all the groups and Ct value of T2 was higher than 33 cycles making the low expressing activation markers T8/T4 undetectable or inconsistent in differentiating the two extreme groups, we made cut off Ct of T2 upto 33 cycle. Accordingly Ct T2 > 33 was considered sample undetermined for T cell response analysis.

Cut off and model of analysis

As a positive control for favourable innate immune response to common upper respiratory tract viral infection we used a cocktail of RNA samples consisting of a mixture 15 SARS-CoV-2 negative but corona virus positive (non COVID-19 corona virus positive) samples (group 6). This cocktail sample was run along with other SARS-CoV-2positive test samples. We determined the average β-actin normalized ΔCt of OAS2 and IL-10 of independent runs in the cocktail samples which was 9 and 12.3, respectively. As average ΔCt of OAS2 in non COVID-19 corona virus infection was 9, we made the cut off 9 for OAS2. ΔCt OAS2 < 9 was considered high expression of OAS2. Samples were marked as 'Impaired IL-10 response' if β-actin normalized ΔCt of IL-10 > 12.5 and ΔCt of OAS2 < 9. "Severity score" for T cell response was derived by the formula (Ct CD69 + Ct GZMB– 2*Ct ZFP36). Score > 12 was considered poor T cell response and high risk.

Outlier detection

Outliers were detected if the values of the severity score in any of the clinical groups were out of the range of mean ± 2SD for the corresponding clinical group. Outlier samples underwent repeat experiments to investigate if there was any technical error. If in any batch of RT-PCR, the melting point temperature for a particular gene was found heterogeneous, test was repeated.

Coefficient of variation (CV) and repeatability test of severity score

For intraday CV, 3 independent cDNAs were made from 4 samples, one from group 4, one from group 5, one from group 6 (non-COVID-19symptomatic of common control) and one from group 2, and PCR reactions were performed on the same plate for the same set of samples.

For inter day CV, cDNA was made in 3 different days and PCR reactions run on the corresponding day with the same set of samples. However, we observed that following repeated freeze thawing, RNA sample started to give higher CT values in every subsequent reaction indicating degradation. However, we observed that most of the samples could tolerate three freeze thaw cycles without qualitative changes in data interpretation.

ELISA

Anti-SARS-CoV-2 spike protein IgG was measured by EDI Quantitative SARS-CoV-2 Spike Protein ELISA kit (Epitope Diagnostics, Inc., San Diego, CA, USA). Plasma, saliva, and VTM samples of COVID-19 patients were used for the detection of anti-SARS-CoV-2 spike protein IgG. 20 µl of standards and 1:40 dilution of plasma samples, 1:5 dilution of saliva, and VTM samples were used as per manufacturer protocol.

Statistical analysis

Mean, standard deviation, median and Pearson correlation were performed. Kolmogorov–Smirnov Test of Normality, Comparison of mean (t-test), significance of Pearson's correlation, significance of association (Fisher exact probability due to small sample size), ODD ration and sensitivity specificity test were analyzed using the following web-based software: MedCalc (https://www.medcalc.org/calc/odds_ratio.php.html) and Social Science Statistics (https://www.socscistatistics.com/tutorials/ttest/default.aspx). Statistical significance was considered if $p \leq 0.05$. For making dot plot web based software Interactive Dotplot was used. ROC curve, AUC, and cut point to maximize Youden's J and test Efficiency was calculated by the web based statistical software: Epitools Epidemiological Calculators. Ausvet. Available at: http://epitools.ausvet.com.au[63].

Data availability

All the relevant Anonymized data will be available from the corresponding author upon reasonable request through data transfer agreements approved by the stakeholders.

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Author contributions

JP conceived and designed the study, data analysis, data interpretation, drafting manuscript; YR executed experiments, data analysis, manuscript editing, AK assisting experiments; NS, AN, VJ, AS management and tracking of COVID-19 samples; MS, FP,AK,AS Sample collection, telephonic interview of patients; RKP,VJ,OS,AK, HL clinical data collection; MS critical views, advising, manuscript editing; All authors manuscript revising and editing.

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Competing interests

JP and YR have a pending patent application to Indian Patent office. Application number: 202321017746; Title: "An invitro method and a kit for determining the severity of COVID-19 outcome". Other authors do not have any competing interest.

Additional information

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