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Combination of exhaled volatile organic compounds with serum biomarkers predicts respiratory infection severity

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ABSTRACT

Objective: During respiratory infections, host-pathogen interaction alters metabolism, leading to changes in the composition of expired volatile organic compounds (VOCs) and soluble immunomodulators. This study aims to identify VOC and blood biomarker signatures to develop machine learning-based prognostic models capable of distinguishing infections with similar symptoms.

Methods: Twenty-one VOCs and fifteen serum biomarkers were quantified in samples from 86 COVID-19 patients, 75 patients with non-COVID-19 respiratory infections, and 72 healthy donors. The populations were categorized into severity subgroups based on their oxygen support requirements. Descriptive and statistical analyses were conducted to assess group differentiation. Additionally, machine learning classifiers were developed to predict disease severity in both COVID-19 and non-COVID-19 patients.

Results: VOC and biomarker profiles differed significantly among groups. Random Forest models demonstrated the best performance for severity prediction. The COVID-19 model achieved 93% accuracy, 100% sensitivity, and 89% specificity, identifying IL-6, IL-8, thrombomodulin, and toluene as key severity predictors. In non-COVID-19 patients, the model reached 89% accuracy, 100% sensitivity, and 67% specificity, with CXCL10 and methyl-isobutyl-ketone as key markers.

Conclusion: VOCs and serum biomarkers differentiated HD, COVID-19, and non-COVID-19 patients, and enabled the development of high-performance severity prediction models. While promising, these findings require validation in larger independent cohorts.

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Machine learning; respiratory infections; volatile organic compounds; serum biomarkers; severity prediction

1. Introduction

Volatile Organic Compounds (VOCs) are generated and released continuously from human body[1] due to host cell and microbiota metabolism.[2] VOC composition changes due to metabolic disturbances in the body or tissues caused by different conditions and diseases, creating distinct patterns.[3,4] Previous studies have identified specific VOC profiles for diseases, including lung infections and cancer.[5,6] Recently, VOC analysis has been applied for COVID-19 screening.[7,8] The COVID-19 pandemic led to unprecedented impact on healthcare, economic and social systems worldwide.[9] The necessary features for mass and rapid diagnosis are short time of detection, non-invasive, facile sample collection and low-cost,[10] like analysis of VOCs in exhaled air.[11–14] Moreover, some independent groups presented successful results of COVID-19 detection by electronic nose devices,[15–17] although in most cases analyses were performed comparing COVID-19 with healthy controls without correlations with severity.

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In addition to diagnosis, prognosis of respiratory infections, especially COVID-19, is a principal area of concern for the scientific community, since COVID-19 manifestations are still unpredictable in particular patients.[18] In severe COVID-19 as well as in other respiratory infections, inflammatory pathophysiology is one of the main contributors to morbidity and mortality[19] as a consequence of a massive unregulated inflammatory activation in response to infections.[20] Previous studies demonstrated that elevated serum cytokine profiles directly correlate with pulmonary inflammation, tissue injury and poor prognosis of severe COVID-19.[21,22] Consequently, most biomarkers investigated in COVID-19 prognosis belong to the immune-inflammatory and coagulation pathways.[18] Notably, many cytokines upregulated in COVID-19 are also increased in other respiratory diseases such as severe influenza infection.[23,24]

Although many biomarkers have been previously tested in COVID-19 patients, their exact utility on diagnosis and prognosis remain challenging.[25] Focusing on separate biomarker levels may give only a partial view of a complex disease.[26] Increasing evidence suggests that artificial intelligence approaches based on biochemical parameters can improve the quality of clinical decision-making.[27,28] Especially, machine learning (ML) methods have been recognised as a helpful tool for COVID-19 diagnosis involving VOCs profile[11] and for COVID-19 prognosis involving immunological features.[29]

Our aim is to combine both VOCs and immune-related biomarkers to propose a broader approach in differentiation and severity classification of patients with respiratory infections.

2. Methods

2.1. Participants

A prospective observational study was accomplished from November 2021 to July 2023, with patients from Hospital Clínico Universitario Lozano Blesa (Zaragoza, Spain) approved by Aragon Clinical Research Ethics Committee (CEICA) (PI21/292). All participants gave written, informed consent. The study included 72 HD, 86 COVID-19 and 75 non-COVID-19 patients (SI, section 1). Patients were grouped by illness severity, according to the requirement for oxygen support. Specifically, patients were categorised into four groups: 50 in the severe non-COVID-19 group, 25 in the mild non-COVID-19 group, 35 in the severe COVID-19 group, and 51 in the mild COVID-19 group.

2.2. Measurements

The samples were collected upon hospital admission for patients and at any time for healthy donors (after certification that they did not have any illness at that time or in the seven preceding days). Gas sampling bags were used to collect expired air from individuals. VOCs were analysed by solid phase microextraction associated with gas chromatography-mass spectrometry (SPME/GC-MS). Concentrations of various immune-related biomarkers were measured in patients' serum, using Multiplex Bead Array.

2.3. Statistical analysis

A detailed explanation of statistical, bioinformatic and ML methods is included in SI, section 2. The main tests and analyses used were Chi-square, Fisher exact test, Mann-Whitney-Wilcoxon, Kruskal-Wallis, Principal Component Analysis (PCA), Ward´s and Sperman´s correlation. ML algorithms, including support vector machines (SVM), k-nearest neighbours (KNN), artificial neural networks (ANN), logistic regression (LR) and Random Forest (RF) were employed.

3. Results

3.1. Differences between COVID-19, non-COVID-19, and HD subjects

Detailed clinical and demographic characteristics of participants are shown in Table 1. The average age of HD group is lower because of the profile of Hospital-admitted patients. The smoking status shows statistical differences only for the comparisons with HD. The ICU admissions number was equal in both patients' groups. 66.7% non-COVID-19 and 40.7% COVID-19 patients were assigned to the severe group based on the oxygen support necessity.

3.1.1. Comparison of VOC levels

PCA was performed to identify VOC profiles associated with disease diagnosis (Figure S1). COVID-19 patients showed the greatest heterogeneity in overall VOCs. Methyl isobutyl ketone (MIBK) and *m,p*-xylene were the most informative variables, demonstrating significant differences between the three diagnostic groups (Figure 1a).

Elevated levels of *i*-Octane, styrene, and beta-pinene were found in patients compared to HDs (Figure 1a) while *m,p*-xylene and *o*-xylene levels were downregulated, being *m,p*-xylene significantly higher in COVID-19 than non-COVID-19. 3-Carene, alpha-pinene, and MIBK were upregulated in COVID-19 while MIBK and isopropanol were lower in non-COVID-19 than HD. COVID-19 cases had higher hexane, ethylbenzene, and limonene levels compared with HD. Toluene and propanoic acid were lower in COVID-19. Decane levels were downregulated in COVID-19 compared to non-COVID-19. The heatmap reveals similar pattern of decane and toluene in all groups (Figure S2). Ethanol and isopropanol were significantly higher in COVID-19, showing a similar profile in the heatmap, indicating upregulation in one COVID-19 clade.

3.2. Differences between COVID-19 and non-COVID-19 patients

3.2.1. Comparison of immune-related biomarker levels

The levels of IL-2, IL-8, IL-17, TNF-α, E-selectin (E-sel) and granzyme A (GzmA) were significantly higher in non-COVID-19 than in COVID-19 patients (Figure 1b and figure S3). However, VCAM-1 was significantly elevated in COVID-19. CXCL10, VCAM-1, tissue factor (TF) and thrombomodulin (TM) showed a similar profile on the heatmap (Figure S3), upregulated in some COVID-19 clusters, and downregulated in the most homogeneous non-COVID-19 clade. The patient's severity visualisation in the upper dendrogram shows high heterogeneity for mild and severe patients' distribution.

3.2.2. Correlation profile analysis between serum parameters and VOCs

Correlation between level of compounds was analysed for COVID-19 and non-COVID-19 patients sub-grouped by disease severity (Figure S4). Apparently, different correlation profiles were observed in non-COVID-19 and COVID-19 patients, as well as in mild and severe subgroups. Non-COVID-19 correlation pattern (Figure S4-A) showed more symmetry for severity than COVID-19 (Figure S4-B) indicating that COVID-19 patients had higher differences in correlation profiles depending on severity.

Table 1. Comparison of demographic and clinic characteristics between healthy control, non-COVID-19 and COVID-19 groups. Statistical analysis was performed through Chi-square or the Fisher exact test for count data. The nonparametric Mann-Whitney-Wilcoxon test was used for continuous variables. A p value < 0.05 was considered significant.

	Mean ± sd, median (IQR) or N (%)			*p* value		
	Healthy controls ($n = 72$)	Non-COVID-19 patients ($n = 75$)	COVID-19 patients ($n = 86$)	Healthy controls vs non-COVID-19	Healthy controls vs COVID-19	Non-COVID-19 vs COVID-19
Gender, n (%)						
Female	55 (76.39%)	32 (42.67%)	38 (44.19%)	**<0.001**	**<0.001**	0.972
Age (years)	39.72 ± 11.23	70.83 ± 19.83	66.67 ± 17	**<0.001**	**<0.001**	0.069
Smoking status, n (%)						
Current smoker	3 (4.17%)	13 (17.33%)	11 (12.79%)	**0.014**	**0.032**	0.862
Former smoker	19 (26.39%)	26 (34.67%)	41 (47.67%)	0.131	**0.004**	0.303
Unknown		4 (5.33%)	1 (1.16%)			
BMI (kg/m^2)		29.14 ± 5.81	28.83 ± 6.66			0.530
ICU, n (%)						
Admission		1 (1.33%)	1 (1.16%)			1.000
Unknown		2 (2.67%)				
Lymphocytes (mil/mm^3)		1.28 ± 0.66	1.1 (0.7)			0.600
Oxygen support, n (%)		50 (66.67%)	35 (40.7%)			**0.001**

sd: standard deviation; IQR: Interquartile Range; BMI: Body Mass Index; ICU: Intensive Care Unit.

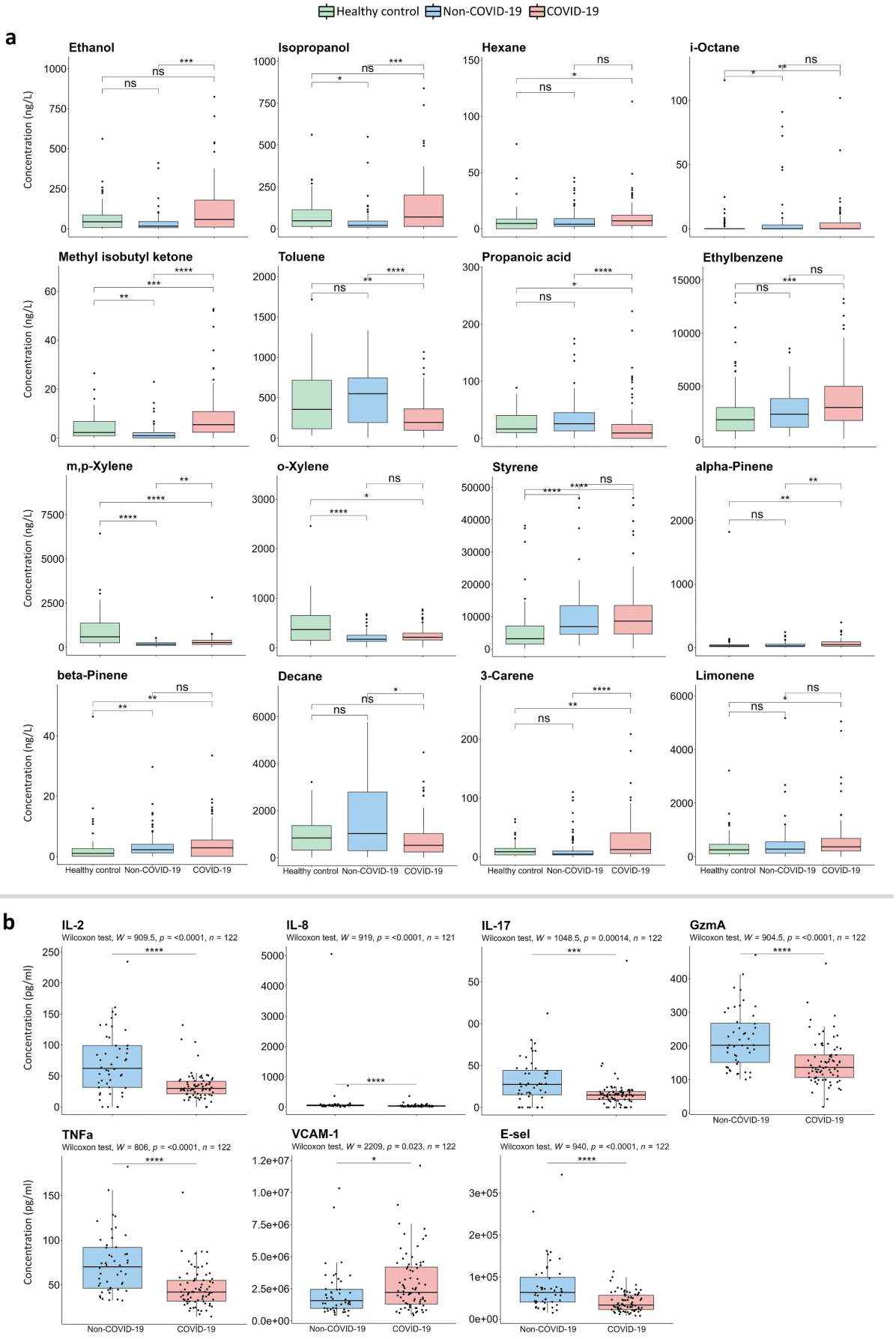

Figure 1. (a) Breath VOC levels comparison between healthy control, non-COVID-19, and COVID-19 subjects. Kruskal Wallis test and multiple Dunn's post-test for comparison between groups. *p* values showing significant post-test comparisons were displayed. (b) Serum parameter levels comparison between non-COVID-19 and COVID-19 patients. Mann-Whitney-Wilcoxon test between groups. A *p* value < 0.05 was considered significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns non-significant.

3.3. VOCs and serum parameters as predictive biomarkers of illness severity

3.3.1. Potential of VOCs and serum parameters in discriminating patients' severity

ROC curves were calculated individually for COVID-19 (Table S1) and non-COVID-19 (Table S2) cohorts, revealing 14 significant compounds ($p < 0.05$) in the COVID-19 group. Figure 2 displays ROC curves for VOCs and serum biomarkers with statistically significant results. IL-6, IL-8, TM, and toluene had p values < 0.001, with AUC values near 0.80, indicating a high individual feature performance.

3.3.2. Development of severity prediction models for COVID-19 and non-COVID-19

Table S3 illustrates the results of severity classification of five ML models combining VOC and serum biomarkers, developed individually for COVID-19 and non-COVID-19 data. These results correspond to the best fit model parameters for each algorithm. Details on the main parameter settings of models were provided in table S4.

For COVID-19, RF model exhibited the highest performance in differentiating mild and severe patients on the testing set, achieving 92.9% accuracy, 100% sensitivity and 88.9% specificity. The accuracy of COVID-19 RF model was significantly superior ($p = 0.018$) than the NIR (the largest class percentage in the data). Similarly, for non-COVID-19 patients, the RF algorithm was the top performer, classifying severity cases with 88.9% accuracy, 100% sensitivity, and 66.7% specificity.

3.3.3. Explanation of random forest models for severity prediction in COVID-19 and non-COVID-19 patients

The top weighted parameters found in Mean Decrease Gini (MDG) plot (Figure S5-A) were CXCL10, MIBK, decane, IL-6, styrene, propanoic acid, von Willebrand factor A2 (vWF A2), ethylbenzene, hexane, *m,p*-xylene and toluene for classifying non-COVID-19 patients into severity groups. The RF model developed on training data was validated on the testing data. Confusion matrix shows results of predictions and real labels comparison (Figure S5-B), giving 88.9% accuracy with one false positive on new data. Figure S5-C illustrates the top 15 features and their SHAP value in the non-COVID-19 severity prediction model. MIBK, CXCL10, ethylbenzene, vWF A2 and GzmB were the most influential compounds in severity prediction for testing dataset. Lower levels of these variables revealed higher SHAP values. This may be interpreted as the probability of a patient developing severe non-COVID-19 increase as those biomarker levels decrease. TF, alpha-pinene and TM showed the opposite trend. To clarify the non-COVID-19 model prediction for individual patients, we performed LIME analysis for mild (Figure S5-D) and severe (Figure S5-E) examples. Severity prediction was correct for both cases. Probability of severe patient prediction was high (0.92). The explanation fit was elevated (0.92 in mild and 0.95 in severe patient), demonstrating good model fitting,

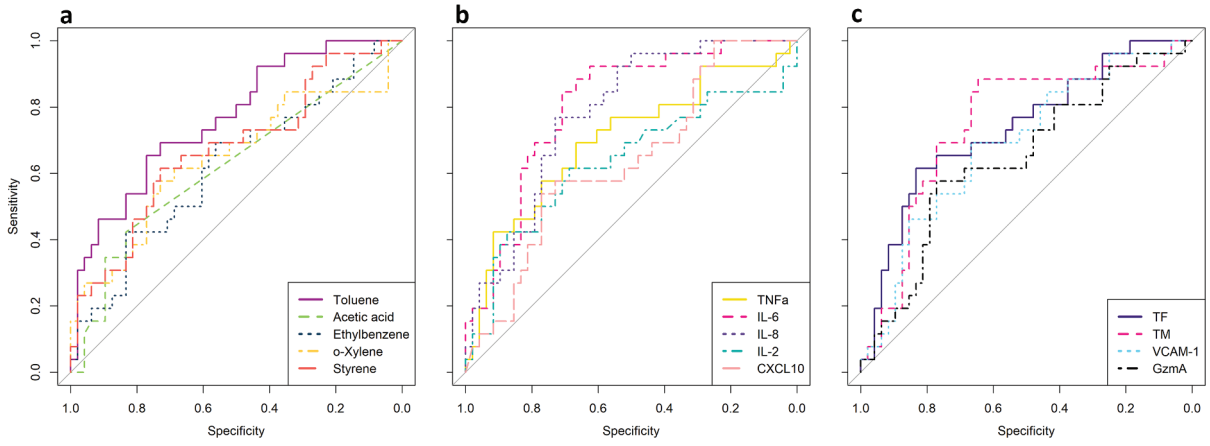

Figure 2. Receiver operating characteristic (ROC) analysis discriminating severe patients from mild individuals belonging to the COVID-19 group. (a) ROC curves of VOCs that shown statistically significant results. (b) ROC curves of cytokines that shown statistically significant results. (c) The rest of ROC curves of serum parameters that shown statistically significant results. A p value < 0.05 was considered significant.

robustness, and high reliability of the probability results in these examples. Propanoic acid level lower than 13.2 ng/L and intercellular adhesion molecule 1 (ICAM-1) level lower than 1156 pg/ml had the greatest influence on mild probability for the case showed in figure S5-D. VCAM1 and 1-butanol had effect in the other direction. In the severe case, vWF A2 levels between 5394 and 7437 pg/ml and *m,p*-xylene higher than 143 ng/L were the most influential compounds for severity prediction.

MDG analysis (Figure 3a) reveals Il-6, IL-8, toluene, TM, TF, styrene, TNF-α, *o*-xylene, ICAM-1 and ethylbenzene as the most important predictors of severity illness in the COVID-19 model development. The confusion matrix showed one false positive, resulting in 92.9% accuracy ($p = 0.018$) when exposed the model to testing dataset. Model performance analysis on test data through SHAP method reveals the same top 5 important variables as in model execution on train data. IL-6, IL-8, toluene, TM and TF showed the highest SHAP values for severe patient prediction, followed by IL-2, ICAM-1, TNF-α, *m,p*-xylene and isopropanol. It is noteworthy that, except for isopropanol and thrombospondin (TSP), all SHAP values increased as the variable levels increase. Hence, higher levels of these compounds mean greater probabilities of being diagnosed as a severe patient. Regarding to LIME analysis, examples of mild and severe case are shown in Figure 3d,e respectively. 0.96 was obtained for explanation fit in mild patient and 0.93 in severe patient, exhibiting high degree of confidence for this severity prediction. For mild case, 1-butanol less than 1.77ng/L and *o*-xylene less than 153ng/L had the greatest influence supporting mild probability. Accordingly, 1-butanol less than 1.77ng/L contradicts the severe probability in the severe case. Likewise, ethylbenzene, propanoic acid and toluene highly support the severe prediction in that patient.

If age, smoking status and gender variables were included in the analysis, the COVID-19 model retained the top three IL-6, IL-8 and toluene as the most significant ones reinforcing their importance in prognosis (Figure S6). On the contrary, age emerged as the most significant variable in non-COVID-19 patients (Figure S7).

4. Discussion

Our findings suggest that integrating VOC analysis from exhaled breath with serum biomarker detection shows promise for developing comprehensive prognostic tools to improve respiratory disease management, especially since they can be assessed non-invasively, providing a practical advantage in clinical settings.

Our results demonstrate distinct VOC profiles on COVID-19, non-COVID-19 patients, and HD, some of which confirm previous studies validating our cohort. For example, hexane and ethylbenzene were elevated in COVID-19 compared to HD samples,[30,31] lower toluene levels were found in COVID-19 patients than in HD[14] and decane levels were higher in non-COVID-19 compared with COVID-19 individuals.[17] In addition to these VOCs, we revealed differences between groups in terpene levels: α-pinene, β-pinene, 3-carene and limonene, which have been previously suggested to be altered in non-infectious lung dysfunction conditions.[32] We also found differences between groups for MIBK, *m,p*-xylene, *o*-xylene and propanoic acid levels, which have not been previously reported. Serum immunomodulators also revealed distinct profiles of COVID-19 and non-COVID-19 patients. The results demonstrating distinct VOC and serum parameter profiles encouraged us to perform our severity study separately for COVID-19 and non-COVID-19 patients.

Consistent with previous results, COVID-19 ROC curves for IL-6,[21,33–35] IL-8,[33,36] IL-2[37,38] or TFN-α[25,38,39] were statistically significant in our study, as well as for CXCL10, TF, TM, VCAM-1 and GzmA. We also found that IL-6, IL-8 and TFN-α were the most important variables in the decision algorithms developed, especially for COVID-19.

Concerning VOC biomarkers, most of studied aromatic hydrocarbons (toluene, ethylbenzene, *o*-xylene, and styrene) showed significant ROC curves, with toluene exhibiting the highest AUC value for distinguishing COVID-19 severity. As expected, those compounds were important variables in RF algorithm training, as well as for test data adjustment on SHAP. Most likely these aromatic compounds proceed from exogenous sources, which, similar to other conditions like COPD, asthma or cancer[40,41] suggest that their presence might contribute to altered lung function during infection. Propanoic acid was very relevant in both patient

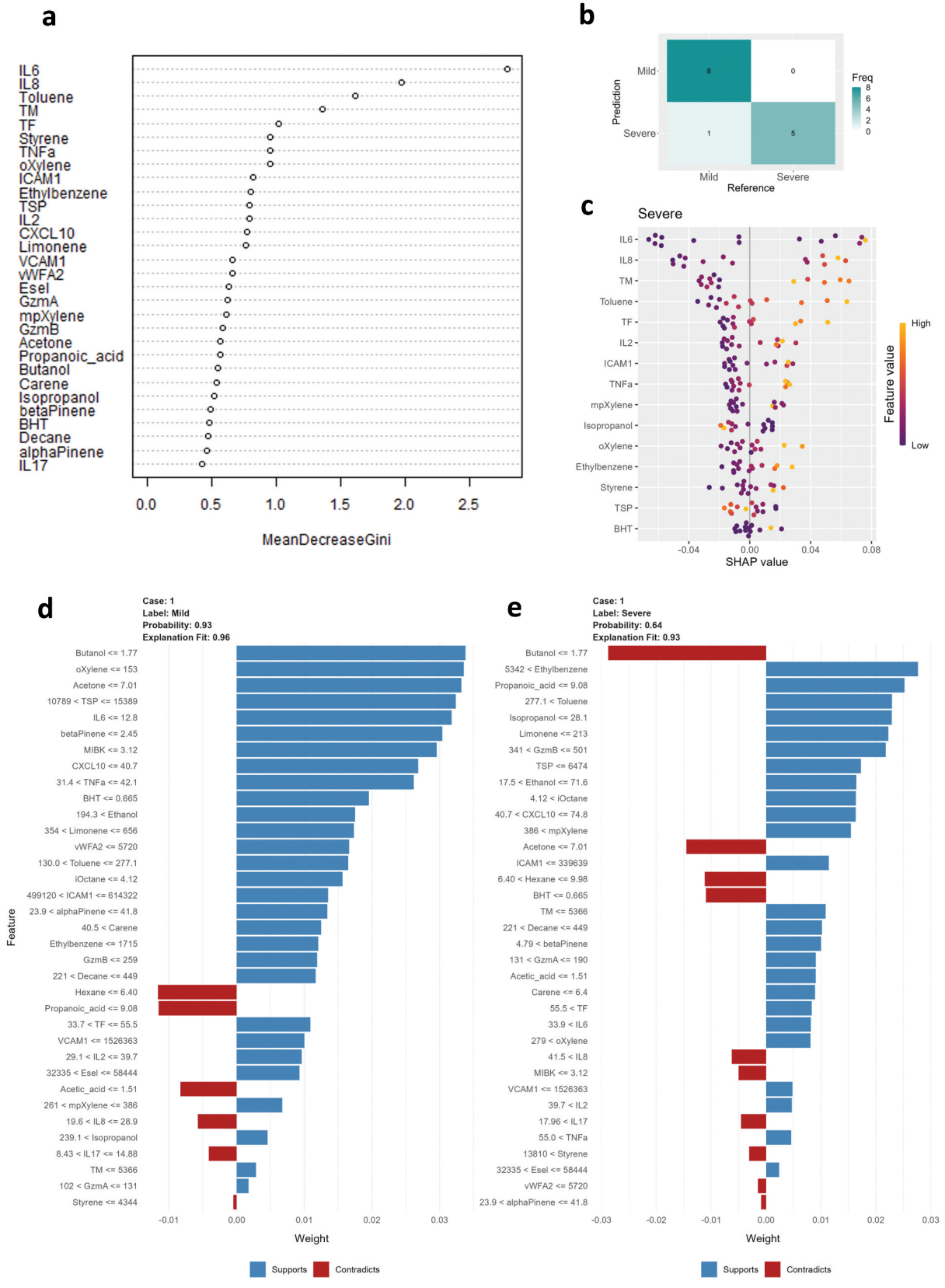

Figure 3. Random forest model results for COVID-19 dataset. (a) Mean decrease Gini (MDG) plot rank the variable importance regarding to disease severity in this classification model. It shows how important each variable is in classifying the training data, expressing how much accuracy the model losses by excluding each variable. The higher MDG value, the higher feature importance in the model. (b) Confusion matrix shows the model performance on test dataset, comparing the model predictions with the actual severity label. (c) Shapley additive exPlanations (SHAP) summary plot quantify the

model training, being the most supporting feature for classifying mild non-COVID-19 and the third in severe COVID-19. Organic acids are mainly formed during bacterial fermentation of carbohydrates and are especially abundant in the human gut.[42] It was suggested dysbiosis of intestinal microbiota as a potential cause for differential propanoic acid production,[43] which might also impact respiratory infection severity.[44]

MIBK demonstrates significant importance in COVID diagnosis, distinguishing between COVID-19 and non-COVID-19 and had high relevance in the non-COVID-19 RF model, being the most important VOC for algorithm execution in both training and testing datasets. Low MIBK levels aid the algorithm in identifying individuals as severe patients. Supporting this finding it was proposed that increased ketone levels result from elevated fatty acid β-oxidation and protein metabolism,[45] processes that are dysregulated in COVID-19 patients.[46] A recent study identified acetone as predictive VOC for disease severity and death outcomes.[12] Accordingly, acetone was one of the most weighted severity predictors in our LIME descriptions of mild COVID-19 and severe non-COVID-19.

Decane is also a crucial feature for the non-COVID-19 severity decision algorithm. Kamal et al. concluded that exhaled breath concentrations of long-chain alkanes correlated positively with nasal concentrations of antiviral cytokines in patients with pulmonary infections.[47] Conversely, our SHAP results for non-COVID-19 indicate a higher probability of severe cases with lower decane levels, possibly due to the diverse range of diseases within the non-COVID-19 group.

It is noteworthy that 1-butanol emerges as the most significant feature influencing severity decisions in COVID-19 LIME examples. In contrast, this variable did not hold the same level of importance during the algorithm's development. This observation underscores the critical importance of validating models with new data not employed in algorithm training. Furthermore, our findings highlight the relevance of retaining features that may not appear useful in individual analyses.

We also investigated the prognostic capacity of a combination of biomarkers for both COVID-19 and non-COVID-19 patients through artificial intelligence for early identification of patients at high risk of severe illness and optimise clinical decision-making. Consequently, ML algorithms, such as the RF model utilised in this study, are particularly valuable for analysing extensive healthcare data and making predictions.[48] However, their black-box nature poses challenges to comprehend their decision-making process. To enhance its interpretability, we employed SHAP and LIME methods to explain global and individualised predictions. Developing transparent and robust models holds great significance for patient classification, disease monitoring, treatment optimisation, and efficient resource allocation. In conclusion, our study reveals for the first time the prognostic value of VOCs profile when combined with immune-related biomarker signature in respiratory infections. We propose this novel point of view to develop decision algorithms that can improve the severity prediction.

A main limitation of this study is the small sample size and diagnostic heterogeneity in the non-COVID-19 group, which may hinder result generalisation. It is important to note that differences in sample size of mild and severe groups may affect the statistical power and the likelihood of detecting significant results. The high number of analysed features pose a risk due to overfitting, leading to good performance on training data but not on new data. It should be noted that despite strong test results, this study is exploratory. To achieve reliable, generalisable results, larger clinical datasets and independent sample testing are needed to support our findings.

variable importance on the test dataset. SHAP values indicate the change magnitude in log-odds (probability of being severe patient). Points coloured by purple-orange gradient indicates the original value for each compound and patient. (d, e) Local interpretable model-agnostic explanations (LIME) show individualised predictions for two examples of (d) mild patient and (E) severe patient. It shows the probability of being classified as mild (d) or severe (e), and explanation fit for each specific patient. Bar length represent how much each variable supports (blue) or contradicts (red) the classification into mild (d) or severe (e). VOC units: ng/L. Serum parameter units: pg/ml.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author's contributions

Conceived and designed the experiments: EMG, JP, JRP. Performed the experiments: PE, MPD, EM, SLG, ARL. Analysed the data: MME, GP, PE, RSP. Contributed reagents/materials/analysis tools: EMG, JP, JRP, RSP. Wrote the paper: PE, EMG, JP.

Data availability statement

The data that support the findings of this study are available from the corresponding authors, [EMG and PE], upon reasonable request.

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