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Early View

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A Biomarker Assay to Risk-Stratify Patients with Symptoms of Respiratory Tract Infection

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Take Home Message: The RALI-Dx assay is a biomarker-based approach to emergency department triage for patients with respiratory illness and is superior to conventional strategies. This diagnostic test will help to optimize the utilization of scarce healthcare resources.

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Abstract

Background: Patients who present to an emergency department with respiratory symptoms are often conservatively triaged in favour of hospitalization. We sought to determine if an inflammatory biomarker panel that identifies the host response better predicts hospitalization in order to improve the precision of clinical decision-making in the emergency department.

Patients and Methods: From April 2020 to March 2021, plasma samples of 641 patients with symptoms of respiratory illness were collected from emergency departments in an international multicentre study: Canada (n=310), Italy (n=131), and Brazil (n=200). Patients were followed prospectively for 28 days. Subgroup analysis was conducted on confirmed COVID-19 patients (n=245). An inflammatory profile was determined using a rapid, 50-minute, biomarker panel: Rapid Acute Lung Injury Diagnostic (RALI-Dx), which measures IL-6, IL-8, IL-10, sTNFR1, and sTREM1.

Results: RALI-Dx biomarkers were significantly elevated in patients who required hospitalization across all three sites. A machine learning algorithm that was applied to predict hospitalization using RALI-Dx biomarkers had an area under the receiver operating characteristic curve of 76±6% (Canada), 84±4% (Italy), and 86±3% (Brazil). Model performance in COVID-19 patients was 82±3% and 87±7% for patients with a confirmed pneumonia diagnosis.

Conclusions: The rapid diagnostic biomarker panel accurately identified the need for inpatient care in patients presenting with respiratory symptoms, including COVID-19. The RALI-Dx test is broadly and easily applicable across many jurisdictions and

represents an important diagnostic adjunct to advance emergency department decision-making protocols.

Introduction

Symptoms of respiratory tract infection are a common cause of emergency department (ED) visits. From 2018-2019 in Canada, more than 200,000 ED visits were associated with respiratory illness.[1] This demand is traditionally increased in the seasonal flu months and has been further complicated in the COVID-19 pandemic,[1] where ED visits for respiratory illness have dramatically increased to the point of repeatedly overwhelming health care systems worldwide. Current diagnostic tests that focus on identifying the underlying etiologic viral agent are useful, but are unable to assess the severity of disease and do not capture the *host inflammatory response*, thus failing to measure the biological indicators of patients that require inpatient treatment[2]. Thus, there is a critical need to develop diagnostic tools that more accurately determine which symptomatic patients have an exaggerated underlying host inflammatory response and should be considered for inpatient treatment following ED presentation.

To assess whether a biomarker panel could provide insight into the host inflammatory response and subsequent need for hospitalization, we tested the RALI-Dx (Rapid Acute Lung Injury Diagnostic; SQI Diagnostics, Toronto, Canada) assay. This is a rapid (<50 minute), multiplexed immunoassay that quantifies interleukin-6 (IL-6), IL-8, IL-10, soluble tumour necrosis factor receptor-1 (sTNFR1) and soluble triggering receptor expressed on myeloid cells-1 (sTREM1). Individually, these immune activation markers have been correlated with a heightened host response to respiratory tract infections, including clinical deterioration and the need for mechanical ventilation in countries with high COVID-19 infection rates.[3—11] To further evaluate the inflammatory profile provided

by RALI-Dx, we applied a novel machine learning approach, XGBoost, to the biomarker output to derive an algorithm that predicts the need for hospitalization that would theoretically help to guide decision-making in the ED.

This study presents the RALI-Dx biomarker results in patients who presented to an ED with symptoms of respiratory infection from 2020-2021 in three countries: Canada, Italy, and Brazil. RALI-Dx biomarkers measured at clinical presentation were compared to disposition from the ED and illness severity (i.e., hospital admission, intensive care unit (ICU) admission and/or mortality) during the 28-day follow-up period. We then describe the development of a novel machine learning algorithm based on the RALI-Dx biomarker results generated in Canada followed by validation on patients in Italy and Brazil. Importantly, we also provide the accuracy of the algorithm in patients with confirmed COVID-19 infection across the three international cohorts.

Methods

Additional details regarding assays, model development and statistical methods are provided in the Supplemental Methods.

Patient Population and Data Source: We conducted a prospective international multicentre study of adult patients presenting to the ED. Inclusion criteria were symptoms of respiratory illness, age 18 years or older, and provision of informed consent. The study comprised three cohorts with identical inclusion criteria: Canada cohort (n=310) of patients presenting to a University Health Network (UHN; Toronto,

Canada) ED from April to October 2020, and an additional set of patients that tested positive for COVID-19 at UHN from November to December 2020; Italy cohort (n=131) of patients presenting to the ED of Cittá della Salute e della Scienza di Torino Hospital-Molinette Site (Turin, Italy) from April to May 2020; and Brazil cohort (n=200) of patients presenting to the ED of Hospital São Lucas-PUCRS (HSL-PUCRS; Porto Alegre, Brazil) from January to March 2021. Controls: healthy health care workers at Cittá della Salute e della Scienza di Torino Hospital-Molinette Site (Turin, Italy) provided samples that served as controls for this study.

Participant Details: A whole blood sample was collected via venipuncture as part of routine blood work during initial evaluation. Demographic details were collected alongside standard vital signs and hospitalization metrics. Hospitalization decisions were made according to each institution's standard procedures. All patients were followed for 28 days after their ED visit via medical records and/or phone call. Patients who withdrew from the study at any time were excluded from analysis. The COVID-19 status of each patient was confirmed using a nasopharyngeal reverse transcription (RT)-PCR swab for the presence of SARS-CoV-2. All studies were reviewed and approved by the Research Ethics Board of each institution (UHN: REB #20-5225, Turin: Comitato Etico #CS2/139, Porto Alegre: CAAE #39181420.0.0000.5336). The study was registered as NCT #04750369.

RALI-Dx Assay: A 5-plex immunoassay (RALI-Dx) was developed based on previous lung injury studies[12] and included the following protein markers: IL-6, IL-8, IL-10, sTNFR1, and sTREM1.

RALI-Dx Model Development: The RALI-Dx biomarker predictive algorithm model was developed using the Extreme Gradient Boosting (XGBoost) algorithm.[13] To ensure that the model was appropriately trained to determine the level of care required, all Canada cohort cases were independently reviewed and adjudicated by two clinicians who were blinded to the RALI-Dx results to determine completion of data and the need for outpatient versus inpatient care (i.e., hospitalized for >72 hours and/or required significant clinical intervention, such as intravenous therapies and/or supplemental oxygen or mechanical ventilation). Any discrepancies between clinicians were resolved via paired consensus.

Statistical Analysis: All analyses were conducted using Stata (StataCorp, TX, USA), GraphPad (GraphPad Software, CA, USA), SPSS Statistics (IBM Corp, NY, USA), Python Programming Language (Python Software, DE, USA), or R statistics.

Results

Patient Characteristics

In the Canada cohort, 310 patients were recruited from the ED with symptoms of respiratory illness. Patients were predominantly male (58%), Caucasian (57%), and presented with shortness of breath (51%) (Table 1). 51% of patients were admitted to

hospital following their ED visit, and 5% of the cohort required care in an ICU within the four-week period after initial presentation (Figure 1). The 28-day mortality rate was 3.5% (Figure 1). Characteristics of the Italy (131 patients) and Brazil (200 patients) cohorts are presented in Table S1 and Figure S1. The levels of RALI-Dx panel biomarkers in each patient population are summarized in Table 1 and Table S1.

RALI-Dx Biomarker Results for Patients with Symptoms of Respiratory Tract Infection

In healthy patients, the plasma levels of RALI-Dx biomarkers were below RALI-Dx detection limits for IL-6, IL-8, and IL-10, and at 637pg/mL and 174pg/mL for sTNFR1 and sTREM1, respectively (Table S5). All RALI-Dx biomarkers were significantly elevated in the plasma of patients who were hospitalized with respiratory illness (Figure 2). Median sTNFR1 and sTREM1 plasma levels were approximately two-fold higher in hospitalized patients (Figure 2). Biomarker levels were lowest in patients who were discharged from the ED for outpatient follow-up care and highest in those requiring care in an ICU at any point during the 28-day follow-up period (Figure 2). Importantly, all RALI-Dx biomarkers were significantly elevated in patients who died during the 28-day follow-up period (Figure 2). Consistent with the Canada cohort, significantly elevated plasma levels of RALI-Dx biomarkers were also observed in patients who were hospitalized in Italy and Brazil (Figure S2).

Univariate logistic regression analysis of RALI-Dx biomarkers shows that each biomarker significantly predicts the decision to hospitalize (Table S4); sTNFR1 and sTREM1 were strong univariate predictors with area under the receiver operating

characteristic curve (AUROC) values of 77% and 70%, respectively (Table S4). Similar trends were observed in the patient cohorts from Italy and Brazil (Table S4). Univariate cut-offs based on Youden's J statistic for each biomarker were: 15pg/mL (IL-6), >0 (IL-8), 7pg/mL (IL-10), 1339pg/mL (sTNFR1), and 316pg/mL (sTREM1) for the Canada cohort. Notably, for patients discharged from the ED with undetectable levels of IL-10 (<7pg/mL), specificity was 100%.

A Model for Host Inflammatory Biomarker Assessments in the ED

The Canada cohort was used to develop a RALI-Dx predictive model, using a tree-based machine learning algorithm called XGBoost. The algorithm determines the probability that a particular patient requires hospitalization using numerous decision-trees comprised of the individual RALI-Dx biomarkers. Patients who required outpatient monitoring versus hospitalization (i.e., inpatient care required) was used as the model classifier. The Canada cohort was randomly partitioned 80:20 for training and testing, and 5-fold cross-validation was performed in the training dataset. The Italy and Brazil cohorts were then used as external test datasets for the RALI-Dx model. Modeling results indicated that all five biomarkers in the RALI-Dx panel were required by the XGBoost algorithm, with SHAP (Shapley Additive Explanations) values >0 for each marker.

As a generalized model to predict hospitalization for any patient with symptoms of respiratory illness, the RALI-Dx model had an AUROC of 82% and 76%, in the training and test (Canada) datasets, and an AUROC of 84% and 86% in the Italy and Brazil test datasets, respectively (Table 2). For comparison, the commonly used clinical model

based on CRB-65 (confusion, respiratory rate, blood pressure and age) had significantly inferior performance of 70% ($p=0.00010$) and 61% ($p=0.017$) in the training and test (Canada) datasets, and 77% ($p=0.024$) and 66% ($p<0.0001$) in the Italy and Brazil cohorts (Table 2).

We investigated the performance of the RALI-Dx model to correctly predict the need for hospitalization in critically ill patients. Based on biomarker levels in the ED, the RALI-Dx model correctly reported an increased probability of hospitalization, and correctly predicted the need for hospitalization in 74% (39/53) of patients who required care in an ICU during the 28-day follow-up period across the three cohorts. Moreover, for high-risk patients that died during follow-up, the accuracy of the RALI-Dx model to predict inpatient care upon ED presentation was 89% (39/44), and was the same for patients that died within seven days of ED presentation (89% (17/19)).

RALI-Dx Model Applicability to COVID-19 and Pneumonia

For patients with a confirmed COVID-19 diagnosis, the plasma levels of RALI-Dx biomarkers were significantly elevated in patients who required hospitalization compared to those that were safely discharged from the ED (Figure 3). There were no significant differences in severity (hospital admission, ICU admission, mechanical ventilation, and death) in the COVID-19 patient cohorts (Table S2).

We then evaluated the RALI-Dx biomarker algorithm to predict the need for inpatient care in patients with COVID-19. There were 245 confirmed COVID-19 patients included

in the study across the Canada, Italy, and Brazil cohorts (Table 2). The AUROC of the RALI-Dx model for disposition decision in COVID-19 patients was 82%. This represents a significant 12% improvement over the clinical CRB-65 approach (70%) ($p<0.0001$; Table 2).

In the Canada cohort, there were n=50 patients with a confirmed community-acquired or COVID-19 pneumonia diagnosis. In this subgroup, the RALI-Dx model predicted the need for hospitalization better than or equally well to known scoring systems for pneumonia patients, namely: CRB-65 and PSI (pneumonia severity index) (Table 3). RALI-Dx and PSI significantly outperformed CRB-65 ($p=0.009$ (PSI vs. CRB-65), $p=0.005$ (RALI-Dx vs. CRB-65)). Similar trends in predictive performance were observed for pneumonia patients that required care in an ICU (AUROC: 72±11% (CRB-65), 72±10% (PSI), 79±15% (RALI-Dx)) or that died during the 28-day follow-up period (AUROC: 72±20% (CRB-65), 91±6% (PSI), 89±8% (RALI-Dx)).

Discussion

In this prospective, multi-site, international observational study, we evaluated the ability of the RALI-Dx immunoassay to augment patient assessments in the ED. We developed a novel, machine learning approach to analyze the RALI-Dx biomarker results and demonstrated a dramatic improvement over the current standard-of-care for ED assessments in all jurisdictions studied. For any patient that presented to an ED with symptoms of respiratory illness, the RALI-Dx model predicted the need for inpatient care with an AUROC of 82% and 76% in training and test datasets from Canada, and

84% and 86% in the Italy and Brazil cohorts, respectively. Notably, this model is also specifically applicable to patients with COVID-19 (AUROC = 82%) and pneumonia diagnosis (AUROC = 87%). It is important to note that this rapid assay is applicable in any respiratory illness regardless of underlying viral etiology. This is of relevance to the current pandemic, any future variants and waves, and any future respiratory pandemics, as the assay is directed to the host response to the viral illness and not the identity of the infecting virus itself.

Respiratory illnesses are a common cause of ED visits and hospital admissions worldwide each year. One of the most widely used clinical assessment tools in the ED to assess severity of respiratory illness, the CRB-65 score, does not precisely assess the host inflammatory response. As a result, patients are conservatively triaged in favour of admission for inpatient monitoring, which potentially admits many more patients to the ward and ICU, and places undue stress on the health care system. This issue has been immensely exacerbated by the COVID-19 pandemic which has imposed a crushing burden on hospitals worldwide.

The host inflammatory response to respiratory illness and, in particular, the pulmonary inflammatory response, represents a common pathway leading to severe disease. This has been shown to been especially true during the COVID-19 pandemic, with latent class analysis identifying a distinct subclass of COVID-19-related acute respiratory distress syndrome (ARDS) having an inflammatory phenotype.[14] Thus, the ability to rapidly quantify multiple inflammatory biomarkers upon presentation is of great benefit

to ED teams in deciding hospital admission, irrespective of the etiology of pulmonary disease.

We and others have previously identified the RALI-Dx biomarkers as critical markers of lung injury during isolated lung assessments.[12,15,16] Importantly, the results of this study confirm that these early markers of acute lung injury and ARDS are well-suited to assess the extent and spectrum of host responses to any respiratory infection. The immunoassay is comprised of IL-6, IL-8, and IL-10—three interleukins which serve as important mediators of inflammatory processes—and sTNFR1 and sTREM1, which indicate TNFα signalling and neutrophil and macrophage activation, respectively. During the COVID-19 pandemic, several additional reports have confirmed the prognostic value of these inflammatory biomarkers and thus support the plausibility of the RALI-Dx concept.[3—11] Observational research has shown that biomarker-based approaches can predict adverse outcomes and mortality in patients with respiratory tract infection.[17] Taken together, these biomarkers provide an objective biological assessment of the host immunological response profile. As such, a rapid diagnostic panel could convey timely, meaningful information to the ED clinician in order to enhance the accuracy of safe and expedited patient assessment and disposition.

In an effort to interpret complex clinical data, artificial intelligence and machine learning techniques have been applied to many aspects of health care. Recently, the XGBoost algorithm has been applied to clinical features to develop models of COVID-19 severity.[18—21] However, those findings have primarily been reported by single-centre or

retrospective studies and do not include cytokines and inflammatory biomarkers, and are therefore unable to capture the extent of the host response. The RALI-Dx biomarker model was developed using a cohort of ED patients from Canada who presented with symptoms of respiratory infection during a period of low COVID-19 infection positivity rates. To test the validity and generalizability of this approach and cohort, the model was validated in external test sites in Italy and Brazil. With consistent performance results across all test datasets, we have shown that the RALI-Dx biomarker model is generalizable across respiratory illnesses and jurisdictions. Importantly, the accuracy of the model was maintained across geographical sites with diverse demographics (i.e., age, ethnicity), clinical practices, COVID-19 burden (Italy and Brazil sites were tested during a peak wave in their respective pandemics) and possible COVID-19 variants.

Existing clinical prognostic tools that are widely used in the ED to determine patient disposition, such as CRB-65 or PSI, generally indicate the safety of outpatient management by estimating mortality risk;[22] however, there are a number of limitations to these approaches that include: the need for an a priori pneumonia diagnosis, generalizability to or specificity in different respiratory illnesses (i.e., COVID-19), the number of input variables needed and/or scoring complexity, and reduced memorability.[23—25] These limitations were confirmed in our study as CRB-65 scores performed poorly compared to the RALI-Dx biomarker-based approach with respect to assessing the need for hospitalization in a generalized population of ED patients with respiratory symptoms. Interestingly, CRB-65 performance in the external cohorts was lower in Brazil compared to Italy and may be attributed to the age dependency and poor

performance of CRB-65 in COVID-19 patients.[26,27] For patients with a confirmed pneumonia diagnosis, RALI-Dx and PSI scoring approaches performed equally well in predicting hospitalization, ICU care, and death; however, the RALI-Dx assay offers a more pragmatic assessment approach as it only requires a single blood sample, provides results in less than an hour and, most importantly, can be applied to suspected pneumonia patients *prior* to diagnosis confirmation.

While the final decision on hospital admission and length of stay can be affected by medical, functional, psychosocial factors, and patients' and relatives' preferences,[28,29] the RALI-Dx biomarker test provides a novel approach to patient disposition assessments that enhances precision of decision-making over existing strategies. Importantly, the RALI-Dx biomarker test provides the probability that a patient requires hospitalization.

There are limitations to this study. The observational study design enabled the description of inflammatory biomarkers and association with patient disposition, but does not allow for evaluation of potential cause-effect relationships between biomarkers and clinical outcomes. While the results of our study are encouraging, a randomized, prospective trial in a diverse patient population would be ideal to fully understand the true impact of biomarker algorithms on ED triage decisions. The results of this study demonstrate the generalizability of an inflammatory biomarker approach to patient prognostication for any respiratory illness; however, supplemental studies that address RALI-Dx performance in additional subpopulations may uncover the relative contribution

of each biomarker for a given pathogen. Though the RALI-Dx inflammatory biomarkers alone were prognostic for the severity of respiratory illness, other biomarkers that were not assessed in this study (i.e., procalcitonin and proadrenomedullin), in addition to approaches that include a larger suite of available clinical parameters and AI-guided ED assessment, are targets for future studies.

In conclusion, a predictive model using a rapid inflammation diagnostic immunoassay (RALI-Dx) represents a valuable tool to accurately assess the individual patient's host inflammatory response in patients presenting to an ED with respiratory illness. This pragmatic assay will augment the precision of clinical decision-making: to admit patients who are likely to develop severe illness, and safely discharge those on a milder course to recover outside of the hospital, thereby better managing limited health care resources. RALI-Dx measures the host inflammatory response in patients with any respiratory illness and, therefore, will be broadly applicable for patients with COVID-19, its variants, as well as any future respiratory pandemic.

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Conflict of Interest Disclosure: SK serves as Chief Medical Officer of Traferox Technologies and receives personal fees from Lung Bioengineering, outside the submitted work. AS, MC, LdS, BW, JV and SK are inventors of a patent licensed to SQI Diagnostics. The inventors fully adhere to a number of policies in place at University Health Network that ensure academic integrity and management of potential conflicts of interest between authors and industry partners. For the purposes of regulatory application, SQI Diagnostics provided funding to support Brazil data collection and the work performed by authors DRM, FOF, CRRS, LSMdS, FMBT, and MHJ, but remained arms-length and blinded from the data analysis included in this study. All other authors (SH, TM, SCJ, AM, JW, BTC, AAS, SHM, JM, ML, ML, SB, KH, MN, MC, MG, EL, KCK, TM and SS) report no conflicts of interest.

Manuscript Tables:

Table 1: Canada patient characteristics at ED baseline

	All Patients
Number of Patients	310
Mean Age (SD) - Years	55 (18)
Male (%)	180 (58%)
Race/Ethnicity	
White/Caucasian (%)	178 (57%)
Black (%)	21 (7%)
South Asian (%)	16 (5%)
East Asian (%)	11 (4%)
Hispanic/Latino (%)	10 (3%)
Mean BMI (SD)	27.4 (7.9)
COVID-19+ (%)	25 (8%)
Respiratory Symptoms	
Fever (%)	75 (24%)
Sore Throat (%)	59 (19%)
Dyspnea (%)	157 (51%)
Chest Pain (%)	87 (28%)
Loss of Taste (%)	24 (8%)
Loss of Smell (%)	19 (6%)
Myalgia (%)	99 (32%)
Fatigue (%)	178 (57%)
RALI-Dx Biomarker Levels	
IL-6 pg/mL (Median [IQR])	12 [0-53]
IL-8 pg/mL (Median [IQR])	0 [0-0]
IL-10 pg/mL (Median [IQR])	0 [0-0]
sTNFR1 pg/mL (Median [IQR])	1194 [770-2407]
sTREM1 pg/mL (Median [IQR])	315 [186-563]

Legend: SD=standard deviation; BMI=body mass index; IQR=interquartile range; IL-6=interleukin-6; IL-8=interleukin-8; IL-10=interleukin-10; sTNFR1=soluble tumour necrosis factor receptor 1; sTREM1=soluble triggering receptor expressed on myeloid cells 1.

Table 2: Prediction of inpatient treatment using RALI-Dx biomarkers or CRB-65 for all patients with respiratory symptoms

	Canada		**Italy**	**Brazil**	**COVID-19**
	Training Dataset	Test Dataset	Test Dataset	Test Dataset	All Patients
Number of Patients	248	62	131	200	245
Model AUROC (SD)					
RALI-Dx	82±1%	76±6%	84±4%	86±3%	82±3%
CRB-65	70±1%	61±7%	77±5%	66±4%	70±3%
p-value	0.00010	0.017	0.024	<0.0001	<0.0001

Legend: AUROC=area under receiver operating characteristic curve; SD=standard deviation.

Table 3: Prediction of hospitalization using RALI-Dx, CRB-65, or PSI for patients with confirmed pneumonia diagnosis in the Canada cohort

	CAP	**COVID-19 Pneumonia**	**Combined**
Number of Patients	25	25	50
AUROC (SD)			
RALI-Dx	92±8%	82±14%	87±7%
CRB-65	70±14%	68±15%	68±10%
PSI	87±10%	84±11%	86±7%

Legend: CAP=community acquired pneumonia; PSI=pneumonia severity index

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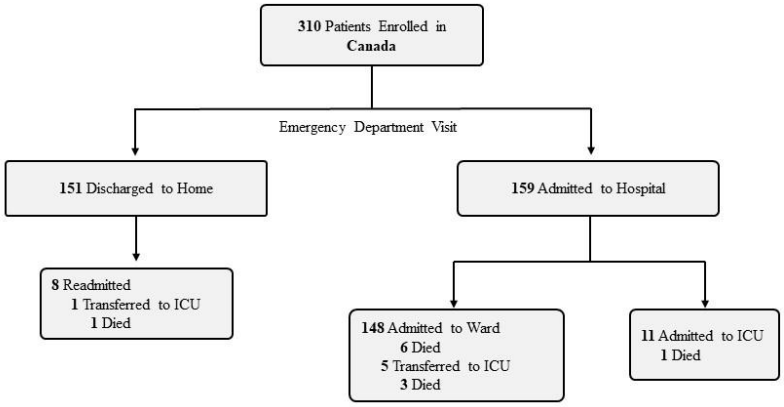

Figure 1: Schematic of patient outcomes following ED presentation.

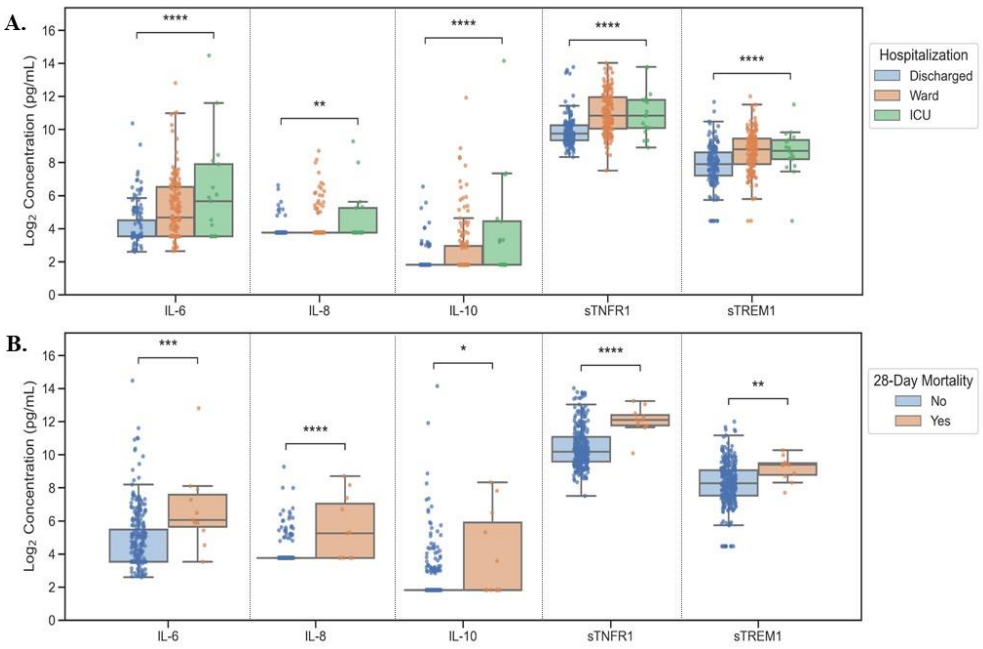

Figure 2: RALI-Dx biomarkers are associated with (A) level of care provided (Kruskal-Wallis) and (B) 28-day mortality (Mann-Whitney U) in the Canada cohort. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

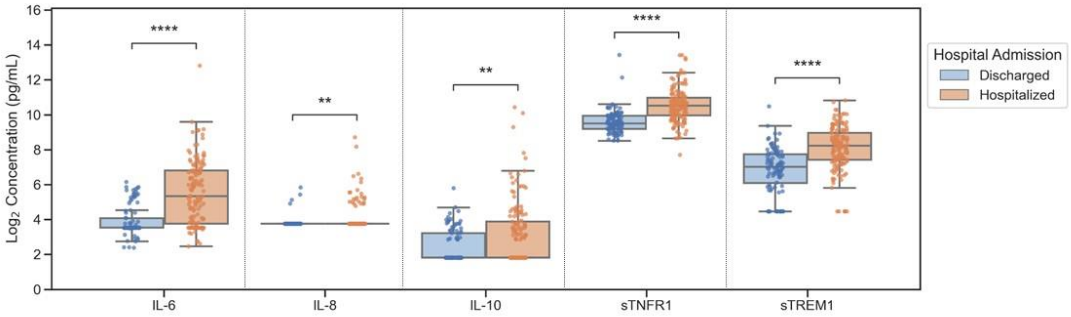

Figure 3: RALI-Dx biomarkers are elevated in hospitalized COVID-19 patients from Canada, Italy, and Brazil. Box and Whisker plots for COVID-19 patients (n=245) that were discharged (blue) or hospitalized (orange) following ED presentation for: IL-6, IL-8, IL-10, sTNFR1, and sTREM1. Mann-Whitney U test p-values are indicated within each graph (**p<0.01,***p<0.001 ****p<0.0001).

Supplementary Materials For:

A Biomarker Assay to Risk-Stratify Patients with Symptoms of Respiratory Tract Infection

Table of Contents:

Supplemental Methods

RALI-Dx Assay: Whole blood samples were collected in the ED and sent to the clinical lab for plasma processing. Samples were stored at -80°C, then thawed overnight at 4°C and diluted 1:1 in assay diluent before testing. 60uL of diluted plasma was loaded on a custom 96-well microtitre plate that contained a standard curve and a high and low positive control derived from the reference standard for each RALI-Dx biomarker. World Health Organization (WHO) reference standards were used for IL-6, IL-8, and IL-10, and a Quantikine ELISA standard (R&D Systems, MN, USA) was used for sTNFR1 and sTREM1. Protein concentrations for each biomarker were determined by quantitative immunofluorescence using the automated sqidlite™ system (SQI Diagnostics, ON, CAN). RALI-Dx analytical validation, including detection capability (i.e., limits of detection and quantification) have been completed (Supplemental Table S3).

Model Development: The Canada cohort was randomly partitioned 80:20 for training (n=248) and testing (n=62); the Italy (n=131) and Brazil (n=200) cohorts were used for external testing. Model performance was assessed using the area under the receiver operating characteristic curve (AUROC) with the null hypothesis that the AUROC was 50%. AUROC significance testing of RALI-Dx model versus the CRB-65 model was determined by Bootstrapping with 10,000 iterations and the null hypothesis that CRB-65 outperforms the RALI-Dx model.

Post-Hoc Model Assessments: The number of patients who developed severe illness (i.e., required ICU care, or died during the 28-day follow-up) were assessed for correctly being predicted to require hospitalization based on the model results from the ED blood sample. Analyses were completed using the optimal probability threshold derived from the Canada cohort (i.e., probability of admission >45%). The RALI-Dx model was evaluated on the COVID-19 positive cohort for model performance (AUROC).

Statistical Analysis: Descriptive statistics of patient enrollment characteristics were evaluated using Chi-squared, Fisher's exact, or Mann-Whitney U tests as appropriate to determine patient factors associated with clinical outcomes. For logarithmic graphs, protein concentrations below the lower limit of detection (LLOD) were assigned a value of 0.5*LLOD in the corresponding figures. Protein distributions followed a non-parametric distribution and were assessed using Mann-Whitney U or Kruskal-Wallis tests where appropriate; multiple comparisons were made using Dunn's correction of the Kruskal-Wallis test. The predictive ability of the individual biomarkers was assessed using AUROC, with the null hypothesis that the AUROC was 50%.

Supplemental Tables

Table S1: Patient characteristics at ED baseline for the external validation cohorts

	Italy Cohort	Brazil Cohort
Number of Patients	131	200
Mean Age (SD) - Years	60 (20)	48 (18)
Male (%)	54 (42%)	94 (47%)
COVID-19+ (%)	56 (43%)	142 (71%)
Respiratory Symptoms		
Cough (%)	56 (43%)	128 (64%)
Fever (%)	75 (57%)	115 (58%)
Sore Throat (%)	11 (8.4%)	86 (43%)
Dyspnea (%)	68 (52%)	107 (54%)
Chest Pain (%)	28 (21%)	72 (36%)
Loss of Taste (%)	23 (18%)	63 (32%)
Loss of Smell (%)	16 (12%)	62 (31%)
Myalgia (%)	··	144 (72%)
Fatigue (%)	··	170 (85%)
RALI-Dx Biomarker Levels		
IL-6 pg/mL (Median [IQR])	6 [0-71]	12 [0-51]
IL-8 pg/mL (Median [IQR])	0 [0-0]	0 [0-0]
IL-10 pg/mL (Median [IQR])	0 [0-0]	0 [0-12]
sTNFR1 pg/mL (Median [IQR])	1064 [704-2173]	838 [610-1386]
sTREM1 pg/mL (Median [IQR])	261 [150-470]	170 [101-306]

Table S2: Outcome severity and RALI-Dx biomarker levels of COVID-19 patients

	Canada COVID-19 Patients	Italy COVID-19 Patients	Brazil COVID-19 Patients	p-value
Number of Patients	47	56	142	-
Hospitalized (%)	27 (57%)	40 (71%)	78 (55%)	0.10
Required ICU Care (%)	4 (9%)	8 (14%)	22 (15%)	0.48
Mechanical Ventilation (any) (%)	3 (6%)	7 (12%)	17 (5%)	0.53
Invasive MV (%)	3 (6%)	1 (2%)	17 (5%)	0.06
Non-invasive MV (%)	··	6 (11%)	··	··
28-Day Mortality (%)	4 (9%)	7 (12%)	18 (13%)	0.73
IL-6 pg/mL (Median [IQR])	13 [0-54]	19 [0-66]	22 [0-66]	0.33
IL-8 pg/mL (Median [IQR])	0 [0-0]	0 [0-0]	0 [0-0]	0.33
IL-10 pg/mL (Median [IQR])	0 [0-0]**	0 [0-6]**	8 [0-14]	<0.0001
sTNFR1 pg/mL (Median [IQR])	1049 [661-1628]	1127 [748-2088]	1006 [702-1553]	0.24
sTREM1 pg/mL (Median [IQR])	287 [123-535]	189 [127-378]	199 [107-338]	0.18

Legend: MV=mechanical ventilation. p-values are reported as Chi square test for hospitalization, ICU care, mechanical ventilation and mortality; Kruskal-Wallis test p-values are reported for protein measurements. Note that (*) indicates a Kruskal-Wallis test multiple comparisons significant difference versus the Brazil cohort (**p<0.01).

Table S3: RALI-Dx performance characteristics

	IL-6	sTNFR1	IL-8	IL-10	sTREM1
ULOQ (pg/mL)	3318	10057	10354	1538	11989
LLOQ (pg/mL)	18.6	118	28	11	238
LOD (pg/mL)	5.1	17	27	7	44

Legend: ULOQ=upper limit of quantification; LLOQ=lower limit of quantification; LOD=lower limit of detection.

Table S4: Univariate logistic regression results for RALI-Dx biomarkers to predict hospitalization following ED presentation

	Canada Cohort		Italy Cohort		Brazil Cohort	
	AUROC	95% CI	AUROC	95% CI	AUROC	95% CI
IL-6	69%	63-74%	80%	72-88%	82%	76-88%
IL-8	57%	53-60%	58%	48-67%	57%	49-65%
IL-10	60%	55-64%	62%	53-72%	67%	59-75%
sTNFR1	77%	72-82%	84%	78-91%	88%	83-93%
sTREM1	70%	64-76%	67%	58-76%	84%	78-89%

Table S5: RALI-Dx biomarker levels (pg/mL) measured in n=20 healthy control subjects

	Median [IQR]
IL-6	0 [0-0]
IL-8	0 [0-0]
IL-10	0 [0-0]
sTNFR1	637 [600-847]
sTREM1	174 [75-288]

Supplemental Figures

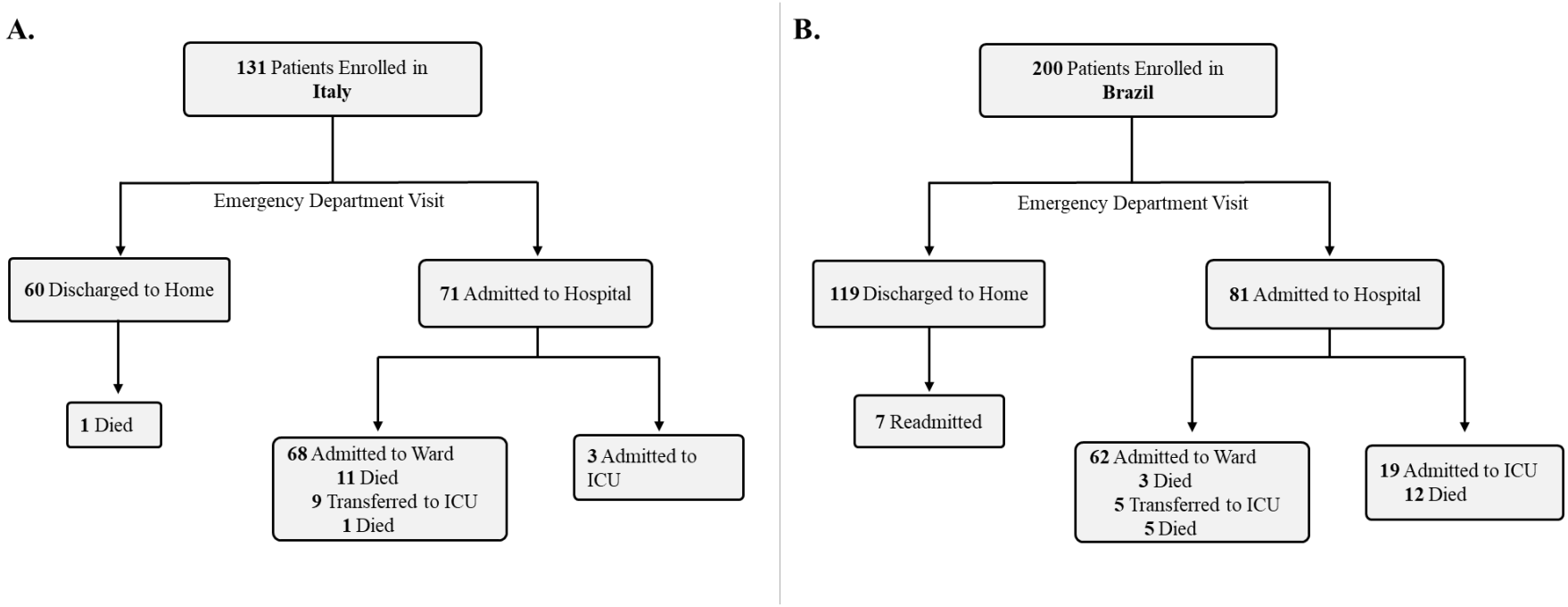

Figure S1: Patient outcomes following ED presentation in **Italy** (A) and **Brazil** (B).

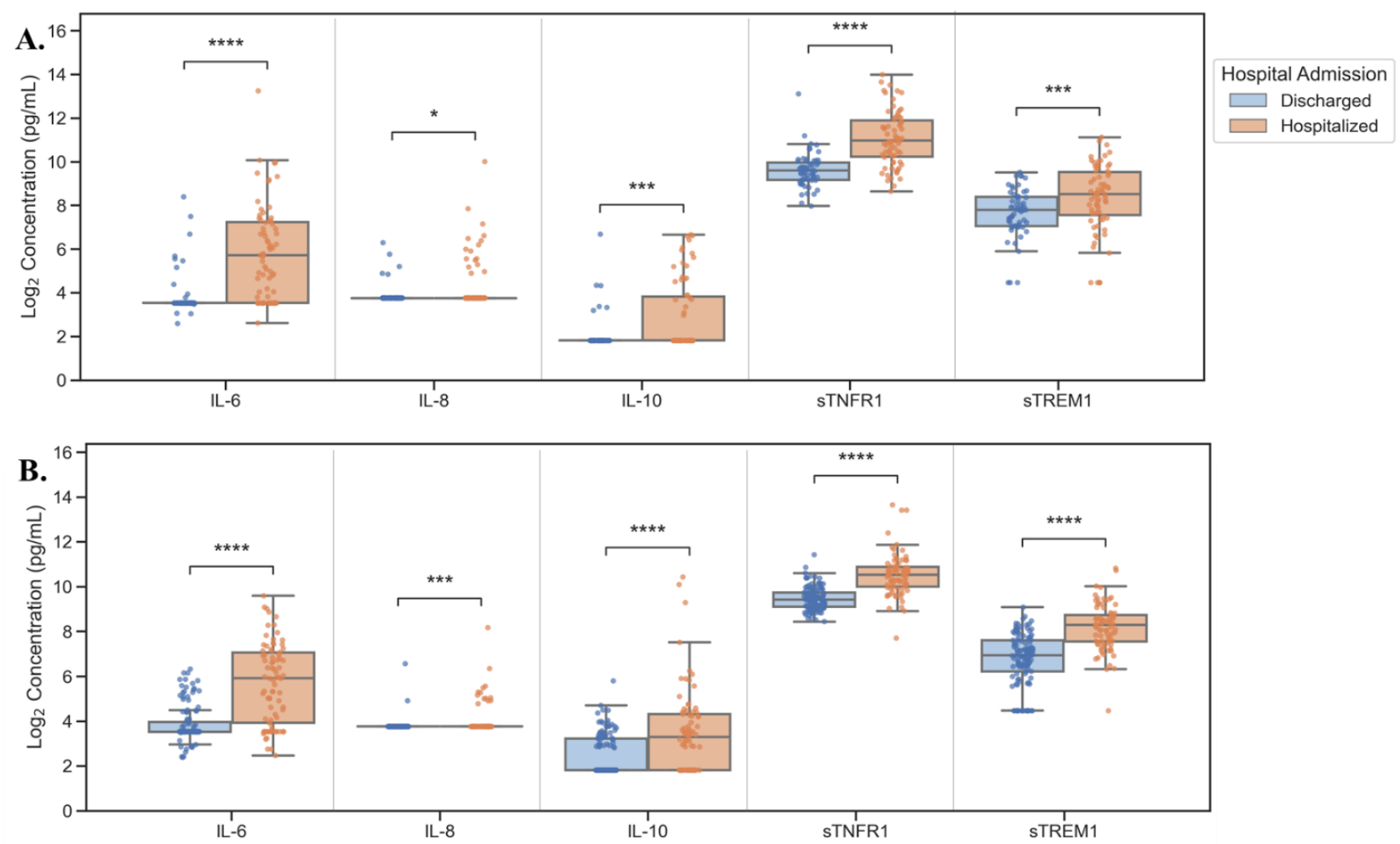

Figure S2: RALI-Dx biomarkers are elevated in hospitalized patients. Box and Whisker plots for patients that were discharged (blue) or hospitalized (orange) following ED presentation for: IL-6, IL-8, IL-10, sTNFR1, and sTREM1 in Italy (A, n=131) and Brazil (B, n=200). Mann-Whitney U test p-values are indicated within each graph (*p<0.05, **p<0.01, ***p<0.001 ****p<0.0001).