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COMMENT

The COVID-19 impact on the scientific production on the 25 main death causes according to world region

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Coronavirus Disease (COVID)-19;
Pandemic;
Health-Related Problems;
Paperdemic

The Coronavirus Disease (COVID)—19, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), affected millions of people and caused the deaths of thousands of individuals worldwide. In this chaotic scenario, the scientific community turned to this topic, aiming to discover diagnostic strategies, treatments, and vaccines. Until April 2021, there were more than 130,000 articles related to the COVID-19 in the PubMed Database. Even though the COVID-19 is an emerging disease, several other health-related problems (HRPs) are responsible for determining global health.[1] Unfortunately, due to the COVID-19 "paperdemic"[2]; several HRPs were less published in 2020.

In this scenario, we described how the COVID-19 impacted on other HRPs publications comparing the numbers of publications between 2020 and 2019 for the 25 Top Causes of Death [World in Data] (Fig. 1). The data search was performed using the follow descriptors: (((health related problems) NOT (coronavirus disease-19 OR coronavirus disease OR coronavirus OR COVID-19 OR COVID19 OR SARS-CoV-2 OR Severe acute respiratory syndrome coronavirus 2))) AND (("1970/12/31″[D*ate* - P*ublication*]: "2020/12/31″[D*ate* - P*ublication*])). The HRPs considered as descriptor were the Top 25 Causes of Death by the "Our World in Data"[3]. In addition, the Top Causes of Deaths according to world area (North America, South America, Europe, Africa, Asia and Oceania)[3,4] were discussed.

We observed that most HRPs, such as cardiovascular disease respiratory infection, lower respiratory disease, dementia, digestive disease, neonatal disorders, diabetes mellitus, liver disease, road injury, homicide, meningitis, nutritional deficiency, protein-energy malnutrition, alcohol use disorder and drug use disorder showed a decreased number of publications in 2020 when compared to 2019, whereas

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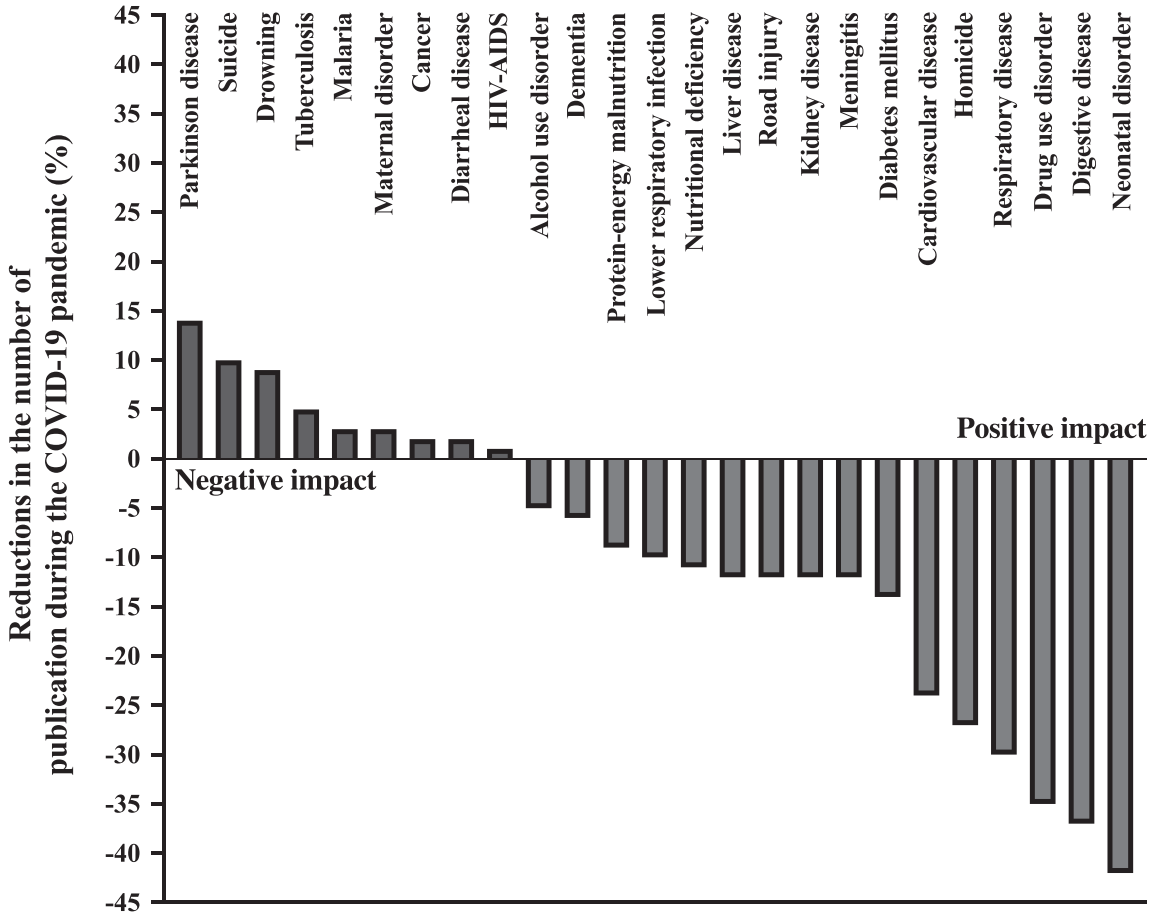

Fig. 1 The COVID-19 Impact on the Scientific Production on the 25 Main Death Causes according to World In Data. Available from: https://ourworldindata.org/causes-of-death.

mental disorders, drowning, Parkinson's disease, malaria, suicide, HIV/AIDS, tuberculosis, diarrheal disease and cancer had more publications in 2020 than in 2019. Even though these 25 HRPs are the deadliest worldwide, the decrease in their scientific production may not have affected equally all the regions, since the top 10 deadliest HRPs in each region are different from each other.[3,4]

It is clear that in highly developed regions, such as North America, Europe, Oceania and Asia, especially China, and even in developing regions, such as South America, especially in Brazil, the impact of the lower publication of cardiovascular disease in 2020 is worrisome, since this is the disease with the highest mortality rate in these regions. All these regions have similar causes of mortality, encompassing cancer, dementia, diabetes mellitus and liver disease, and the decrease in publications about these topics might negatively influence the treatment and prevention of these diseases.

Nevertheless, in underdeveloped regions, like most of the African continent, the causes of mortality are different from those found in the aforementioned regions. For this reason, the decreased publication regarding cardiovascular disease or diabetes mellitus might not have as much impact as the decrease in publications regarding neonatal disorders or HIV/AIDS, which are much more common causes of mortality in these regions. However, publications of only COVID-19 centered research may take an even more brutal toll on this population; hence their main sources of mortality were not focused even before the pandemic, not favoring targeted public health interventions.

Not only has the COVID-19 had a great impact on global health, but also on the scientific community, which has always been able to adapt, seeking efficiency and accuracy. As an emerging disease, several researchers rushed to understand this novel virus, disseminating a great number of papers, which may have even been characterized as a "hype" across the scientific world. Since the novel virus is responsible for more than 160 million confirmed cases and more than three million deaths worldwide, the need for fast information about it is crucial. It is clear that COVID-19 publications have changed the basic structures and focus of publications. For instance, COVID-19 papers underwent review faster than other subjects, culminating in a high rate of retracted publications and a high rate of preprint publications has also been reported. Curiously, before the pandemic, only 2% of the biomedical studies were related to virology whereas during the pandemic, around 10—20% of biomedical papers are COVID-19-related.[5—8] In December 2019 there were thousands of COVID-19 papers published, and many uploaded to preprint, prioritizing rapid dissemination over peer-to-peer review. Undoubtedly, research on a novel disease epidemiology, mechanism of infection, treatment, prevention and origin are of the

utmost importance, for better knowledge and, consequently, the ability to decrease the number of deaths and confirmed cases. However, most non-COVID-19 research was suspended.[8]

Out of the 25 conditions with highest mortality rate, only nine had more publications in 2020 when compared to 2019. Even though new information regarding the COVID-19 is necessary to attenuate the pandemic, it is not prudent to focus only on the COVID-19. For example, papers related to cardiovascular disease, which is the condition with the highest mortality rate in the world, decreased 24%, which could prevent further development of its understanding, especially in highly developed and in developing regions. Also, underdeveloped regions might suffer even more from the decreased number of publications regarding their most common causes of mortality, which were not the focus of studies even before the pandemic, thus taking an even worse toll.

Thus, the COVID-19 deserves, indeed, special attention by the scientific community, however, several other diseases might be neglected, which can also compromise the health worldwide and bring as many deaths as the COVID-19.

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EDITORIAL

Making IGRA testing easier: First performance report of QIAreach QFT for tuberculosis infection diagnosis

Tuberculosis (TB), with around 1.4 million deaths, is one of the most important leading killer for infectious diseases in the world. Moreover, a quarter of the world population is estimated to live with TB infection (TBI).[1,2] TBI is considered the reservoir of active TB, therefore, diagnosing and treating TBI is a crucial component of the End TB Strategy to achieve TB control.[3] So far, the diagnosis of TBI is based on tuberculin skin test and on interferon (IFN)-γ release assays (IGRAs) such as T-SPOT. TB and Quantiferon-TB Plus (QFT-Plus).[4] However, costs, complexity, and supply chain requirements hamper the implementation of current tests for TBI in decentralized settings. Therefore, there is an urgent need for accelerating the scale-up of such assays to inform test-and-treat algorithms for TBI.[5]

The QIAreach QFT test is a novel and simplified version of QFT-Plus. Indeed, it uses a single tube corresponding to the TB2 tube of the QFT-Plus. However, unlike QFT-Plus, the test provides a qualitative result through a fluorescence lateral flow reader, which is transportable, easy to use and does not need highly trained personnel.[6,7] The assay time to result (TTR) is around 20 minutes. Therefore, the QIAreach QFT test has the potentials to be a point-of-care test.

The paper of Fukushima et al[7] aimed to evaluate the accuracy of the QIAreach QFT test for the detection of TBI compared to the commercial QFT-Plus test in both immunocompetent and immunocompromised individuals. The authors evaluate the tests' clinical performance in patients with active TB, used as a surrogate for TBI. Compared to QFT-Plus, which was used as reference standard, QIAreach QFT showed a 99% overall concordance and an optimal accuracy with 100% sensitivity and 98% specificity, similar to QFT-Plus 4. Interestingly, QIAreach QFT also scored positive in samples with IFN-γ level falling in the so-called uncertainty zone of the QFT-Plus.[8] IFN- γ level inversely correlated with TTR, suggesting that the TTR may be used as a surrogate marker for the IFN-γ concentrations. The QIAreach QFT test also scored positive in individuals with CD4 counts $<$200 cells/μl, thus showing potential for immunocompromised individuals.

Multicenter studies are needed to validate these results, and to evaluate the QIAreach in immunocompromised individuals. Moreover, data on precision, repeatability and reproducibility, as well as costs analyses are needed to fully understand the potential of this platform. However, these optimal pilot performances, together with the technical advantages of the test (a single 1 mL of blood is needed), the short TTR, the portability and the multipurpose design of the platform allowing for diagnosing relevant diseases other than TB,[9] make the QIAreach QFT test a promising tool for TBI screening in peripheral settings for easier identification of people eligible for TB preventive therapy. This tool may be useful for TBI screening based on the new suggestions proposed by the WHO, and could support contact investigation to early identify individuals at risk for acquiring TBI also in high TB prevalence countries.[5,10]

The further development and availability of tests for predicting the risk of progression from TBI to active disease will complete the portfolio for maximizing the impact of TB preventive treatment strategies.

Declaration of Competing Interests

PM and LP have nothing to declare. DG is a consultant for QIAGEN and Biomerieux and gave lectures for Diasorin.

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ORIGINAL ARTICLE

First clinical evaluation of the QIAreach™ QuantiFERON-TB for tuberculosis infection and active pulmonary disease

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QIAreach™ Quanti-FERON-TB;
QuantiFERON®-TB Gold Plus;
Active tuberculosis;
CD4 T-lymphocyte;
CD8 T-lymphocyte

Abstract
Objective: 1) to compare the QIAreach™ QuantiFERON-TB (QIAreach QFT) vs. QuantiFERON®-TB Gold Plus assay (QFT-Plus) to detect tuberculosis (TB) infection; 2) to evaluate diagnostic sensitivity of QIAreach QFT using active TB as surrogate for TB infection; 3) to preliminarily evaluate QIAreach QFT in immunocompromised individuals.
Methods: QIAreach QFT measures the level of interferon-γ (IFN-γ) in plasma specimens from blood stimulated by ESAT-6 and CFP-10 peptides in one blood collection tube (equivalent to the TB2 tube of the QFT-Plus). QIAreach QFT was applied to plasma samples from 41 patients with pulmonary TB and from 42 healthy or low-TB-risk individuals.
Results: Sensitivity and specificity of QIAreach QFT vs. QFT-Plus were 100% (41/41) and 97.6% (41/42), respectively; overall concordance was 98.8% (82/83). All samples were measured within 20 min. The time to result of each sample was significantly correlated with IFN-γ level with a natural logarithmic scale (r = -0.913, $p < 0.001$). Seven cases in the active TB group were immunocompromised (CD4 $<200/\mu$L) and tested positive by QIAreach QFT.
Conclusions: QIAreach QFT provides an objective readout with a minimum blood sample volume (1 mL/subject), potentially being a useful point-of-care screening test for TB infection in high-TB-burden, low-resource countries and for immunocompromised patients.

Introduction

Tuberculosis (TB), the world's leading cause of death due to a single infectious agent, *Mycobacterium tuberculosis*, is one of the top-ten causes of preventable death globally.[1] The World Health Organization (WHO) estimates

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that in 2019 alone, around 10 million people suffered from TB and 1.4 million people died from the disease,[1] with one-third of humans having *M. tuberculosis* infection.[2]

Diagnosis and treatment of TB infection are core conceptual elements of the TB elimination strategy,[3-6] as reflected in WHO's emphasis on TB prevention in its End TB Strategy.[1]

Few tests are available to detect TB infection. The century-old tuberculin skin test (TST) is based on delayed type hypersensitivity reaction in the skin upon intradermal injection of purified protein derivative (PPD) from mycobacterial culture. Although TST is still widely utilized, it has several limitations[7-12] that interferon-γ (IFN-γ) release assays (IGRAs) have been developed to overcome.[13] IGRAs are *in vitro* blood assays that measure the levels of IFN-γ released by T lymphocytes stimulated with antigenic peptides of *M. tuberculosis*.[3,4,12-17] Two WHO-endorsed IGRAs are commonly used to detect TB infection: T-SPOT®.*TB* (Oxford Immunotec, Abingdon, UK) and QuantiFERON®-TB Gold Plus (QFT-Plus, QIAGEN, Hilden, Germany).[18,19] The QFT-Plus assay, the fourth generation of QuantiFERON®-TB, is designed to measure IFN-γ released by both CD4 and CD8 T cells.[20,21] To date, both WHO-endorsed IGRA tests need quality laboratory support, potentially limiting their use in peripheral and/or limited resource settings.

Lateral flow immunoassays (LFAs) are portable, easy to use outside specialized laboratory environments, and provide a quick readout, making them ideal point-of-care (POC) tests.[22] QIAGEN has recently developed a new diagnostic test for TB infection, the QIAreach™ QuantiFERON-TB (QIAreach QFT) assay. This novel digital fluorescence LFA uses nanoparticle technology to measure the levels of IFN-γ in plasma released from both CD4 and CD8 T cells, thus eliminating the need for enzyme-linked immunosorbent assay (ELISA).[23] QIAreach QFT, which uses the same test tube as the TB2 tube of QFT-Plus, is an easy-to-use rapid test requiring less instrumentation and blood volume than QFT-Plus. Key characteristics of the QIAreach QFT assay compared to QFT Plus are presented in Table 1.

No previous study has compared the new QIAreach QFT test against the established (FDA-approved and CE-marked) QFT-Plus test in detecting TB infection. Furthermore, the potential role of QIAreach QFT as a diagnostic test for TB infection has never before been evaluated.

The aims of this study were to 1) compare the QIAreach QFT and QFT-Plus tests to detect TB infection; 2) evaluate the clinical performance of QIAreach QFT for detection of TB infection by analyzing plasma samples from patients with active TB disease and healthy or low-TB-risk individuals in a clinical setting; and 3) conduct a preliminary evaluation of the QIAreach QFT test in immunocompromised individuals.

Material and methods

Study subjects

This study was conducted at the Nagasaki Genbaku Isahaya Hospital, a Nagasaki Prefecture-designated TB hospital in Japan. Plasma samples were collected from consenting individuals with active TB (September 2019-October 2020) and from healthy low-TB-risk individuals (August-October 2020). The research protocols for this study were approved by the Institutional Review Board of the Nagasaki Genbaku Isahaya Hospital (approval IRB no.138). Written informed consent was obtained from all recruited subjects.

Clinical and demographic data collected from patients included age, gender as well as key white blood cell count parameters (Table 2). Adult patients aged $\geq$20 years with active pulmonary TB were included in the study if they presented signs and symptoms compatible with TB, imaging (chest radiography and high-resolution computed tomography) compatible with the disease, and met one or both of the following criteria: 1) sputum-culture positive for *M. tuberculosis* and/or 2) TB nucleic acid amplification test-positive specimens tested using polymerase chain reaction or loop-mediated isothermal amplification. Study participants with active TB either had not

Table 1 Comparison of QIAreach QFT and QFT-Plus.

	QIAreach QFT	QFT-Plus
Volume of blood sample and tubes	1 mL, one tube	total 4 mL, four tubes (Nil, TB1, TB2, Mitogen,1mL each)
Stimulation antigens	ESAT-6 + CFP-10+ short peptide CFP-10	TB1: ESAT-6 + CPF-10 TB2: ESAT-6 + CPF10+short peptide CFP-10
Incubation time	16-24 h	16-24 h
Principle of IFN-γ detection	Digital fluorescence lateral flow nanoparticle technology	Enzyme-linked immunoassay; ELISA (colorimetric) system
IFN-γ measurement time and number of samples	Max 20 min/test, 8 tests/eHub	At least 150 min/test 44 samples/kit, 22 samples/plate
Data management	Laptop PC/QIAreach software	ELISA workstation/QFT-Plus software
Instruments for assay	Incubator/centrifuge (not always necessary), eHub	Workstation (plate washer/plate reader)/incubator/centrifuge
Power supply	USB or 100 ~240 volt	100 ~240 volt
Assay handling	Easy	Moderate
Cut-off value	N/A	TB2 (or TB1)-Nil 0.35IU/mL

ELISA: enzyme-linked immunosorbent assay; ESAT-6: early secretory antigenic 6 kDa; CFP-10: culture filtrate protein 10.

Table 2 Characteristics of study participants with active pulmonary TB and healthy low-TB-risk individuals.

Characteristics	Patients with active TB (n = 41)	Healthy / low-TB-risk individuals (n = 42)	p value
Age, years; median (IQR)	82.0 (76.0-89.0)	39.5 (30.75-47.25)	$p < 0.001$
Sex, male; n (%)	27 (65.9)	10 (23.8)	$p < 0.001$
Pulmonary TB; n (%)	41 (100.0)	NA	NA
White blood cell count/μL; median (IQR)	4930 (4395-6965)	5655 (5340-7232.5)*	p=0.17
Lymphocyte count/μL; median (IQR)	1170 (930-1610)	2105 (1662.5-2412.5)*	$p < 0.001$
CD4 cell count/μL; median (IQR)	384 (256-529)	741 (639.5-950)*	$p < 0.001$
CD8 cell count/μL; median (IQR)	222 (148.5-343.5)	516 (358.5-678)*	$p < 0.001$

Data are presented as No. (%), or median (IQR).
TB: tuberculosis; IQR: interquartile range; CD4: CD4+ T lymphocyte in blood; CD8: CD8+ T lymphocyte in blood.
* Data from 26/42 healthy/low-TB-risk individuals.

been treated for TB or had received anti-TB drugs for a maximum 14 days. Healthy low-TB-risk study participants were 1) adults aged 20-65 years, 2) had never taken any anti-TB medication, 3) had no history of contact or exposure to TB, 4) had not lived or stayed in an area or country with a TB incidence rate of $>$50/100,000 for $>$1 month, 5) had no immunodeficiencies such as human immunodeficiency virus infection, malignancy, diabetes mellitus, and treatment with steroids or immunosuppressant drugs.

CD4 and CD8 T-cells in participants' peripheral blood were quantified using flow cytometry as a part of their routine diagnostic management. Assays were performed on CELL-DYN Sapphire Hematology Analyzer (Abbott Co., USA) using proprietary CD3/4/8 monoclonal antibody panels with automated gating.

QFT-Plus assay

For QFT-Plus test, 4 mL of whole blood was taken from all study participants directly into QFT-Plus blood collection tubes. After centrifugation, plasma specimens were harvested and stored at -30°C for later analysis as per manufacturer's guidelines (QIAGEN GmbH, Hilden, Germany). The results of the QFT-Plus test, given as a measurement of IFN-γ, were expressed as IU/mL.

QIAreach QFT assay

One mL of whole blood was taken from all study participants directly into the QIAreach QFT blood collection tube (equivalent to the TB2 tube of the QFT-Plus). Upon centrifugation as per manufacturer's guidelines, plasma was transferred to a microtiter tube and kept frozen at -30°C until testing. Upon thawing the specimens, plasma specimens were centrifuged again at 3000 $\times$ g for 15 min and tested according to the manufacturer's instructions.

Prior to starting the assay, QIAreach-Software-x64-1.1.12.0 was installed on a computer running the Microsoft Windows operating system. A charged eHub, connected to the computer via USB cable, was powered on and the eStick was inserted into the eHub's port. Once connected and turned on, both the eHub and the computer software reported that the eHub was in ready mode. A total of 150 μL of diluent buffer was added to the processing tube. Next, 150 μL of plasma specimen was transferred into the same processing tube. The resulting solution was mixed by pipetting up and down at least four times. A total of 150 μL of this mixture was aliquoted from the processing tube into the sample port of the inserted eStick. The assay began automatically, with the status displayed on both the eHub and the computer upon sensing the mixture. Upon assay completion, the test result (+ or -) and time to result (TTR) were indicated on both the eHub and the software.

Statistical analysis

Data were statistically analyzed using the IBM® SPSS® Statistics V27 for Windows (IBM Corp., USA) and presented as No (%) or median (interquartile range) unless otherwise specified. Sensitivity (positive rate), specificity (negative rate) and overall concordance (proportion of true results overall) of QIAreach QFT were calculated using QFT-Plus as a reference standard. Culture-positive patients were considered the gold standard when evaluating the sensitivity of QIAreach as a tool for detecting TB infection. A sub-analysis was also conducted on samples from immunocompromised patients (CD4 cell counts $<$200/μL). A Mann-Whitney U test is performed for differences of CD4 or CD8 cell counts in the peripheral blood between active TB and healthy low-TB-risk individuals. Linear regression analysis was performed to examine the relationship between the TTR (second) and the IFN-γ levels (IU/mL). A p-value of less than 0.05 was considered statistically significant.

Results

Characteristics of study participants

Clinical information about the study subjects is shown in Table 2. The 41 study participants with active pulmonary TB were median aged 82 years (interquartile range, 76.0-89.0) with median CD4 count: 384 cells/μL (interquartile range, 256-529) and median CD8 count: 222 cells /μL (interquartile range, 148.5-343.5). Both the CD4 and CD8 cell counts of active TB patients were significantly lower than those of the

healthy individuals ($p < 0.001$ for both comparisons). Of the 41 patients recruited, 7 were classified as immunocompromised; differences in cell counts between active TB patients and controls maintained with removal of 7 immunocompromised patients.

Sensitivity, specificity and concordance of QIAreach QFT TB compared with QFT-Plus

Plasma samples from 41 active TB patients and 42 healthy or low-TB-risk individuals were tested. The QIAreach QFT and QFT-Plus ELISA tests were simultaneously conducted on the same samples. Test results are shown in Table 3.

Using the QIAreach QFT assay, 100% sensitivity and 97.6% specificity (95%CI: 92-100% and 88-99%, respectively) were achieved, with overall concordance of 98.8% (95%CI: 94-100% and kappa coefficient = 0.976) (Table 3). Six specimens had uncorrected TB2 tube values without Nil subtraction below 1 IU/ml (ranging from 0.46 to 0.77) on QFT-Plus and all tested positive on QIAreach QFT (Fig. 1.1 A). All specimens tested negative on QIAreach had TB1-Nil and TB2-Nil values below 0.2 on QFT-Plus.

Evaluation of the sensitivity of the QIAreach QFT assay for active TB

Sensitivity of QIAreach QFT for detection of active TB was also 100%. Our assessment of this cohort of plasma samples revealed a single false-positive result from a healthy individual. This false-positive result case had normal CD4 and CD8 cell counts in blood; CD4: 1740/μL and CD8: 711/μL. TB1-Nil and TB2-Nil values measured by QFT-Plus were 0.01 IU/ml and 0.00 IU/ml, respectively, and TTR was 1200 s. In the active TB group, 7 participants aged 70-95 years (median age: 86 years) were immunocompromised (CD4 <200/μL) and tested positive by QIAreach QFT.

Relationship between IFN-γ level of positive sample and time to result

The TTR was examined for all QIAreach QFT-positive results, because TTR is related to the level of fluorescent signal generated in the test. The TTR for QIAreach QFT- positive samples varied from 215-1200 seconds (20 min).

Fig. 1.1 shows the distribution plot of plasma IFN-γ concentrations in IU/ml (presented as uncorrected TB2 values without Nil subtraction) versus TTR for positive samples demonstrating negative correlation between TTR and IFN-γ in TB2 tube. Data transformation of TTR and IFN-γ to a natural logarithmic scale showed high correlation (r = -0.913, $p < 0.001$) between IFN-γ levels and TTR when linear regression analysis was performed (Fig. 1.3).

Six samples testing positive on QFT-Plus had IFN-γ levels (uncorrected and corrected TB2 values) >10 IU/mL (Fig. 1.1 B and Fig. 1.2.B). The TTR for these positive plasma samples, taken from patients aged 71-95 years (median age: 82 years), ranged from 215-305 seconds (median: 245 seconds). The range of the CD4 cell counts for these samples was between 138 and 1270 cells/μL (median: 529 cells/μL), and the CD8 cell counts ranged from 75-1230 cells/μL (median: 312 cells/μL). In comparison, six samples from patients aged 73-92 years (median age: 90 years) that tested positive on QIAreach QFT, with a TTR of 1200 seconds each, had IFN-γ levels on QFT-Plus (uncorrected TB2 values) ranging between 0.46 and 0.77 IU/mL (median: 0.75 IU/mL) (Fig. 1.1 A). Also, these 6 samples had IFN-γ levels on QFT-Plus (corrected TB2 values) ranging between 0.36 and 0.68 IU/mL (median: 0.555 IU/mL) (Fig. 1.2 A) showed positive results by QIAreach QFT. The distribution plot and correlation analysis using plasma IFN-γ values (corrected TB2 values) versus TTR for positive samples showed similar results (r = -0.918, $p < 0.001$) (Fig. 1.2 and 1.4).

The ranges of their CD4 and CD8 cell counts in blood were between 101 and 284 cells/μL (median: 209 cells/μL) and between 66 and 185 cells/μL (median: 115 cells/μL), respectively. Within this group of plasma samples, three came from immunocompromised patients, each with CD4 T-lymphocyte counts <200 cells/μL.

Discussion

This is the first evaluation of a new diagnostic test, QIAreach QFT, in detecting TB infection compared with the QFT-Plus assay and as a screening tool for TB infection.

This new IGRA test is based on digital fluorescence LFA with nanoparticle technology. Similar to the QFT-Plus assay, it measures the levels of IFN-γ secreted from both CD4 and CD8 T lymphocytes in response to stimulation with *M. tuberculosis*-specific antigens, with advantages in use as point-of-care-test.[24]

The QIAreach QFT test exhibited high clinical performance: 100% sensitivity, 97.6% specificity, and 98.8% overall concordance using QFT-Plus as the reference standard. Sensitivity for detection of active TB was also 100%. The specificity and sensitivity of the QIAreach QFT assay reported here are comparable to those previously reported for the QFT-Plus assay. An assessment of the performance of the QFT-Plus assay among active TB patients and healthy individuals in Japan reported 96.2% sensitivity and 96.7% specificity.[25] According to a meta-analysis of 15 published reports, the QFT-Plus assay had a pooled sensitivity of 94% for active TB patients and a pooled specificity of 96% for healthy

Table 3 Diagnostic performance of QIAreach QFT assay using QFT-Plus assay as a reference standard.

	Healthy controls		Active TB		
	QFT-Plus positive	QFT-Plusnegative	QFT-Plus positive	QFT-Plusnegative	Total
Positive QIAreach QFT result	0	1	41	0	42
Negative QIAreach QFT result	0	41	0	0	41
Total	0	42	41	0	83

Sensitivity: 100.0% (41/41); specificity: 97.6% (41/42); overall concordance: 98.8% (82/83).

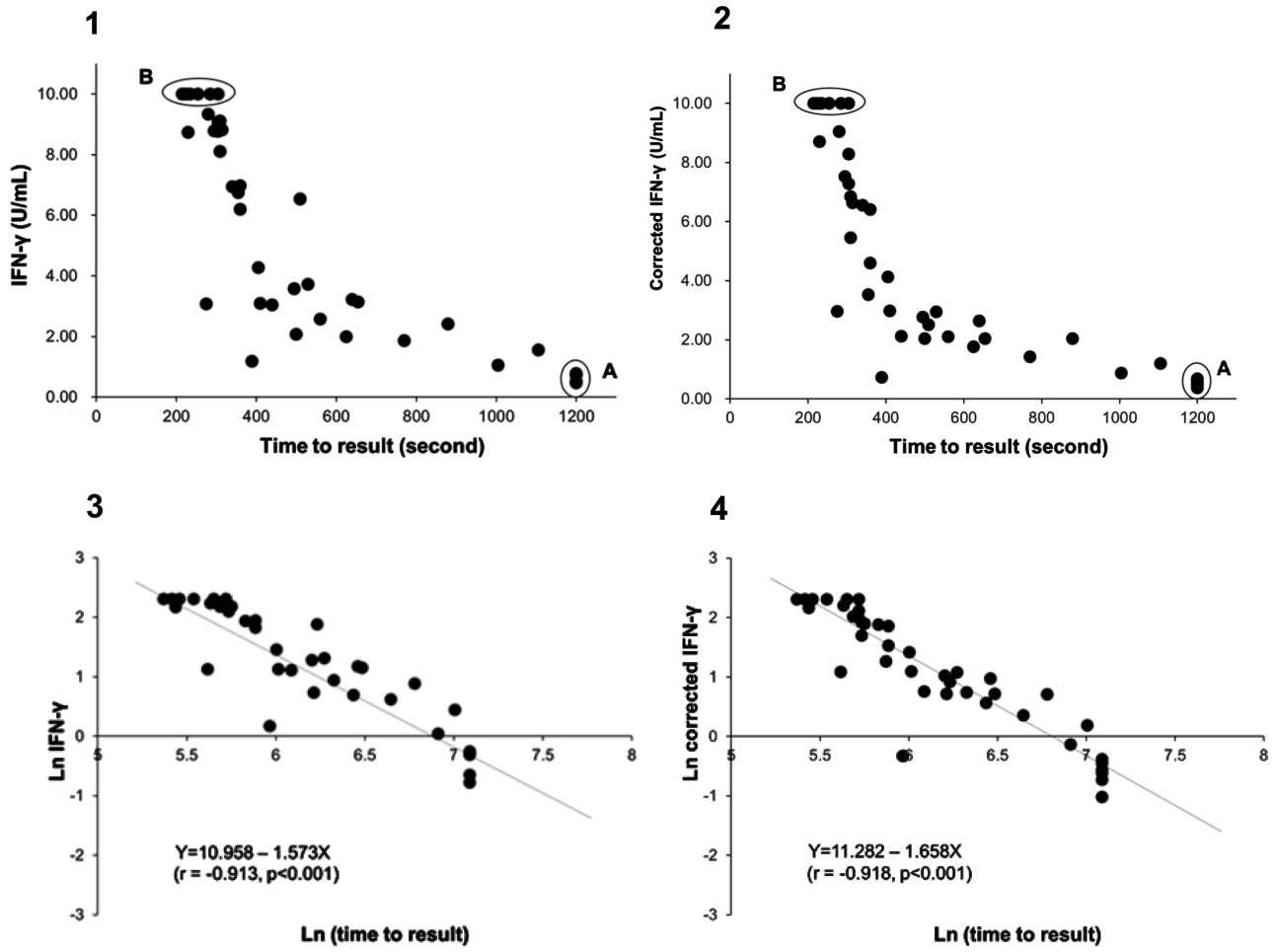

Figure 1 Distribution plot and correlation analysis using plasma IFN-γ values (uncorrected and corrected TB2 values) versus TTR for positive samples (n = 41 active TB patients)

1 — uncorrected values; 2— corrected IFN-γ values; 3— natural logarithm converted uncorrected values; 4— natural logarithm converted corrected IFN-γ values

Fig. 1.1A: Six samples with IFN-γ levels (uncorrected TB2 values) between 0.46-0.77 IU/mL (median: 0.75 IU/mL) and time to result of 1200 s.

Fig. 1.2A Corrected TB2 values ranging between 0.36 and 0.68 IU/mL (median: 0.555 IU/mL)

Fig. 1.1B and 1.2B: Six samples with IFN-γ levels >10 IU/mL (uncorrected and correctedTB2 values) and time to result between 215-305 s (median: 245 s). Data transformation of TTR and IFN-γ of uncorrected and corrected TB2 values to a natural logarithmic scale showed a significantly high correlation (r = -0.913, $p < 0.001$ and r = -0.918, $p < 0.001$, respectively) between IFN-γ levels and TTR by linear regression analysis (Fig. 1.3 and 1.4). Corrected IFN-γ (Fig. 1.2) means IFN-γ levels with Nil subtraction. IFN-γ: interferon-γ; TB: tuberculosis.

individuals.[26] A multicenter investigation into the performance of QFT-Plus test at three U.S. sites and two Japanese sites found the test to have 93.0% sensitivity in adult TB patients.[27]

Notably, the values of the IFN-γ levels shown in Fig. 1.1 and 1.3 are the uncorrected and corrected values of TB2 tubes of the QFT-Plus assay for all the samples tested—i.e., the level of IFN-γ in each TB2 tube without and with subtracting the level of IFN-γ of the Nil tube (background tube). Our results demonstrated that the cut-off point of IFN-γ concentration for QIAreach QFT assay might be similar to that of the QFT-Plus assay (0.35 IU/mL). We did find a statistically significant relationship between levels of uncorrected and corrected IFN-γ in plasma of active TB patients and TTR (natural logarithms conversion of each) with a linear regression analysis (r = -0.913, $p < 0.001$ and r = -0.918, $p < 0.001$, respectively) (Fig. 1.3 and 1.4). This study's results suggest that the higher the IFN-γ level of the sample, the shorter the TTR, which could be used as a surrogate marker of IFN-γ concentration in plasma when using QIAreach QFT assay. Like QFT-Plus, however, QIAreach QFT may have variations in measured IFN-γ values near the cutoff (i.e., 0.2 - 0.7 IU/mL), so there is a relatively high possibility of false negatives and/or false positives for the QIAreach QFT results with a TTR close to 1200 seconds.

Seven cases in the active TB group who were immunocompromised (CD4 <200/μL) returned positive results on QIAreach QFT, suggesting this assay could be considered a

promising digital fluorescence LFA for detecting TB infection among immunocompromised patients. Larger studies on representative cohorts are needed to confirm its performance in those immunocompromised.

Our assessment of this cohort of plasma samples revealed a single false positive from a healthy individual. This was a sole sample with a high triglyceride level (1588 mg/dL) and was turbid following the freeze and thaw processes. Testing on this individual was repeated by obtaining a new sample exhibiting a high triglyceride level (1288 mg/dL) that was not subjected to freeze and thaw processes. A negative result was obtained. Various factors, such as sample viscosity, may have affected the development speed of the sample solution on the nitrocellulose membrane of the LFA system. Of note, the false-positive results of QIAreach QFT assay could be caused by milky plasma as well as autoimmune disease.[28]

The QIAreach QFT assay offers a number of workflow advantages over more complex laboratory-based assays, such as QFT-Plus (Table 1). The QIAreach QFT assay is objective—reporting test results as either positive or negative—and it requires only 1 mL of blood from each patient, compared with 4 mL of blood required for the QFT-Plus assay. In addition, the QIAreach QFT test results can be obtained within a relatively short time of up to approximately 20 min for each specimen analyzed. In contrast, the QFT-Plus test based on ELISA requires at least 150 min to obtain a readout. Moreover, the QIAreach system can be used for a single test or up to eight tests at a time for each eHub being used. The QIAreach QFT testing system and hardware can be used anywhere, like the QIAreach anti-SARS-CoV-2 total test.[23] Implementing the QIAreach QFT testing system does not require any specialized instruments (e.g., an automated ELISA workstation), trained laboratory officers to perform ELISA or a dedicated laboratory space; importantly eHub is battery operated allowing its use in remote areas with limited electricity supply. These features make the QIAreach QFT a suitable and highly attractive test to detect TB infection in decentralized settings.

A TB diagnostic test with these characteristics is of particular value for screening efforts in countries with high TB prevalence.[24] In 2019, TB cases in countries in Southeast Asia and Africa accounted for 69% of the total TB cases worldwide.[1] However, these settings often lack the resources for maintenance and calibration, the infrastructure for complex instrumentation, and the specialized laboratory staff needed for older laboratory-based assays. Many of these low-resource settings that need TB infection screening are outside of major urban centers. The QIAreach QFT test will also be useful for TB infection screening among special groups, including—among others—immigrants,[19] inmates,[29] and children.

The study has clear limitations, including the fact it was conducted in a single center and on a convenience sample (i.e., being a preliminary study, no sample size calculation was performed), thus requiring larger studies to confirm the findings.

Conclusions

In terms of clinical performance, the QIAreach QFT assay displayed 100% sensitivity, 97.6% specificity, and 98.8% overall concordance compared with the QFT-Plus test. This assay is objective, quicker to perform than QFT-Plus, and requires only a small amount of blood (1 mL) per test. The test offers flexibility in that it can be easily performed anywhere and is not restricted to a laboratory environment. This novel assay can be useful in screening for TB infection in high-TB-burden, low-resource countries and, also, for screening of immunocompromised patients. Larger studies are necessary to confirm these preliminary findings.

Authors' contributions

Authors' contributions were as follows: study conception and design (FK, KT), acquisition of data (FK, AK, KA, KT), and analysis and interpretation of data (FK, KT, SN, MH). All authors have contributed substantially to drafting and revising the article critically for important intellectual content. All authors approved the submitted version of the article. K. Fukushima: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. K. Akagi: Data curation, Writing - original draft, Writing - review & editing. A. Kondo: Data curation, Writing - original draft, Writing - review & editing. T. Kubo: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. N. Sakamoto: Formal analysis, Investigation, Writing - original draft, Writing - review & editing. H. Mukae: Formal analysis, Investigation, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. All authors approved the submitted version of the article.

Declarations of Competing interest

None.

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BRIEF COMMUNICATION

COVID-19 Pneumonia and ROX index: Time to set a new threshold for patients admitted outside the ICU

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KEYWORDS
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Abstract
High flow nasal cannula (HFNC) is used to treat acute hypoxemic respiratory failure (AHRF) even outside the ICU and the ROX index (pulse oximetry/fraction of inspired oxygen/respiratory rate) may predict HFNC failure.
Objective: The purpose of this investigation was therefore to verify whether the ROX index is an accurate predictor of HFNC failure for COVID-19 patients treated outside the intensive care unit (ICU) and to evaluate the validity of the previously suggested threshold.
Design: Multicenter study. Retrospective observational analysis of prospectively collected data.
Setting: 3 centres specialized in non-invasive respiratory support (Buenos Aires, Argentina; Bolzano and Treviso, Italy). Patients treated outside the ICU were analysed
Measurements: The variables to calculate the ROX index were collected during the first day of therapy at 2, 6, 12 and 24 hours and then recorded every 24 hours. HFNC failure was defined as escalation of respiratory support to invasive mechanical ventilation (IMV) or death.
Main results: A total of 35 (29%) patients failed HFNC and required intubation. ROC analysis identified the 12-hour ROX index as the best predictor of intubation with an AUC of 0.7916[CI

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95% 0.6905-0.8927] and the best threshold to be 5.99[Specificity 96% Sensitivity 62%]. In the survival analysis, a ROX value <5.99 was associated with an increased risk of failure (p = 0008 log − rank test). The threshold of 4,9 identified by Roca as the best predictor in non-COVID patients, was not able to discriminate between success and failure (p = 0.4 log-rank test) in our patients.
Conclusions: ROX index may be useful in guiding the clinicians in their decision to intubate patients, especially in patients with moderate ARF, treated therefore outside the ICU. Indeed, it also demonstrates a different threshold value than reported for non-COVID patients, possibly related to the different mechanisms of hypoxia.

Introduction

High flow nasal cannula therapy (HFNC) is increasingly used in the management of acute hypoxemic respiratory failure (AHRF), as well as during the outbreak of Coronavirus disease (COVID-19) (1,2,3). In this latter scenario HFNC has been extensively used also outside the Intensive Care Unit (ICU) (2, 4), due to the paucity of ICU beds (5), at least in certain geographical areas (6). Failure of HFNC may cause delayed intubation and increased mortality in patients with ARF (7). ROX index is defined as the ratio of pulse oximetry/fraction of inspired oxygen (SpO_2/FiO_2) to respiratory rate (RR). Roca et al. (8), identified patients at high risk of HFNC failure when this index is <4.88 at 12 hours. This threshold was confirmed also in COVID-19 patients who show, however, an unusually high rate of intubation (9), compared to most of the studies performed in this population (∼30%) (1 Vianello,2 Franco,3 Patel). To the best of our knowledge only one small single centre study was performed (10) outside the ICU and therefore generalizing about a threshold value to predict HFNC success or failure needs confirmation and verification by multicenter trials performed in less "protected environments."

The purpose of this investigation was therefore to verify, in a larger multicenter study, whether the ROX index is an accurate predictor of HFNC failure for COVID-19 patients treated outside the (ICU) and to eventually compare with the previously suggested threshold.

Methods

We performed a retrospective observational analysis of prospectively collected data in 120 patients with ARF due to COVID-19 pneumonia, referring to 3 centres specialized in non-invasive respiratory support (Buenos Aires, Argentina; Bolzano and Treviso, Italy). Patients treated outside the ICU were analysed. The respiratory COVID-19 areas consisted of a former respiratory ward, transformed into an ad-hoc dedicated specialized Respiratory Monitoring Unit. These units provided an active full-day shift run by a fixed group of pulmonologists and with a "reinforced" nurse−patient ratio varying from 1:4 to 1:6 depending on the hospital. Patients with a "do not intubate order" were excluded.

HFNC was initiated with high flows of 50-60 L/min, and adjusting FiO2 to maintain SpO2 between 92-96%. The temperature was targeted according to patient comfort. The patients were monitored by non-invasive measurement of heart rate and blood pressure, oxygen saturation and respiratory rate. FiO2 was gradually reduced keeping the target SaO2. Flow was also gradually decreased according to the patient's tolerance and reduction of respiratory rate (RR). On the other hand, when patients could not sustain SpO2 or reduce RR, they were upgraded to NIV. If the patient's status deteriorated, she/he was transferred to the ICU for endotracheal intubation, at the discretion of the responsible physician. HFNC failure was defined as escalation to invasive mechanical ventilation (IMV) or death. The standard indications for endotracheal intubation (ETI) included the following: respiratory rate (RR) greater than 35 breaths/min, obvious accessory respiratory muscle activity or abdominal paradoxical breathing, progressive increase in $PaCO_2$, hemodynamic instability and inability to protect the airways or inability to obtain saturation greater than 93% with FiO2 greater than 80%.

The switch to NIV or CPAP was not considered as HFNC step-up, since all these 3 methods are considered as non-invasive ventilator support strategies (2 Franco) and the literature has not so far demonstrated superiority of one of these techniques over the others. For this reason, this subset of patients, as well as those passing from NIV to HFNC, was not considered in the data analysis.

Demographic variables and severity scores were recorded at the time of patient admission. The variables to calculate the ROX index were collected during the first day of therapy at 2, 6, 12 and 24 hours and then recorded every 24 hours.

Statistical analysis

The quantitative variables were expressed as mean and standard deviation or median and interquartile range, if the normality criterion, as a result of the Shapiro-Wilk test, was not met. Categorical variables were expressed as frequencies and percentages. Continuous variables were compared using the t-Student or U-Mann Whitney test, as appropriate. For categorical variables, the comparison was made using the chi-square test or the Fisher exact test, as appropriate. To evaluate the accuracy of certain variables for classifying patients who will succeed or fail with HFNC, an analysis of ROC (Receiver Operating Characteristics) curves was made and the area under the curve (AUC) was calculated. The optimal threshold of the continuous variables was chosen to maximize the sum of sensitivity and specificity. According to the cut-off point in the ROC curve analysis for ROX index, Kaplan-Meier curves were used to determine the probability of IMV for patients with a ROX index above the threshold and below the threshold. These curves were compared using the log-rank test. A 2-tailed p-value of less than 0.05 or less was

considered statistically significant. The statistical analysis was performed using R Studio (Version 1.3.1093).

Results

From March to August 2020, 120 of confirmed COVID-19 patients undergoing HFNC fulfilled the eligibility criteria and were included in the statistical analysis. Overall patient characteristics are illustrated in Table 1 S. A total of 35 (29%) patients failed HFNC and required intubation. These patients had higher X-ray consolidations (11) and SOFA. The median time-to-intubation was 2 days (IQR[1-3]). The overall mortality was 9 (7.5%), all in the intubation group. At admission, the median SpO_2/FiO_2 was 155 (IQR[106-190]) and RR was 30.00 (IQR[28-33]). Table 1 shows the accuracy of the ROX index in discriminating HFNC success at 2, 6, 12 and 24 hours.

As shown in the upper part of Fig. 1, the ROC analysis identified the 12-hour ROX index as the best predictor of intubation with an AUC of 0.7916[CI 95% 0.6905-0.8927] and the best threshold to be 5.99[Specificity 96% Sensitivity 62%]. This difference was significantly different when compared to ROX index at 2 hours(AUC 0.6378 p-value = 0.01432) and at 6 hours (0.6648 p-value = 0.001236*)*. In the survival analysis (lower part), a ROX value <5.99 was associated with an increased risk of failure (p = 0008 log $-$ rank test). Interestingly, the threshold of 4,9 identified by Roca as the best predictor in non-COVID patients, was not able to discriminate between success and failure (p = 0.4 log-rank test) in our patients. Among components of the index, SpO_2/FiO_2 had a greater predictive power than respiratory rate.

Discussion

The ROX index has been proposed as a tool to predict HFNC outcome in patients with ARF, mainly admitted to the ICU. In this multicenter study, we have demonstrated that the ROX-12 is also able to discriminate HFNC success from failure in COVID-19 patients, but not with the threshold value proposed by Roca et al., since we have shown better prediction accuracy with a higher threshold (i.e. 5.99). The novelty of this study relies also on the fact that it was performed in patients with less severe hypoxemia treated outside a "protected environment", compared to those treated in the ICU (i.e. baseline SpO2/FiO2 = 155 in our study vs. 104 in Ref. 8)

Previous small single centre studies performed in COVID-19 patients reported a lower value (9, 10) (i.e. 4.95 and 5.40), but this was assessed within the first 6 hours of treatment, suggesting a worse severity of the patients. Nevertheless, it is important to note that during this pandemic HFNC has been largely used outside the ICU and therefore this study may provide useful information in patients with AHRF not needing ICU admission. One may argue that using ROX-12 may delay intubation, however, it has been shown that in these patients most intubations occur between 12 and 24 hrs, and this holds particularly true for patients affected by moderate ARF. Indeed, the reported difference with the validation study by Roca (8), may also be related to the mechanisms of hypoxemia in pneumonia for COVID-19 being different from those of "de novo" ARF (12), where the index was first validated. In particular, we have identified different phenotypes in patients with COVID-19 acute respiratory failure, such as "classical" ARDS, lung injury plus high dead-space related to emboli/diffuse microthrombi or normal lung with embolism (12).

We have shown that the ROX-12 index had a greater predictive value than respiratory rate alone, in contrast with Blez et al. (13) that reported the best accuracy for this latter parameter. That study was however performed in a very small group of patients with a surprisingly low flow (10 L/min). The setting of flow may also drive the changes in respiratory rate, via a modification in end-expiratory lung volumes (14).

This study has some limitations. Firstly, it is a retrospective analysis, but it was based on prospectively collected data. Because of the retrospective nature, standardization of intubation was not decided a priori, but since the 3 hospitals have cooperated in previous common studies, the local guidelines for intubation were very similar.

Table 1 Accuracy of SpO_2/FiO_2, RR and ROX index in discriminating HFNC success at 2, 6, 12 and 24 hours.

	AUC ROC	95% CI	Threshold	Sensitivity	Specificity
At 2 hours					
SpO_2/FiO_2	0.61	0.48-0.74	158	43	85
RR	0.64	0.51-0.78	26	50	83
ROX	0.64	0.52-0.77	5.1	32	98
At 6 hours					
SpO_2/FiO_2	0.66	0.54-0.78	167	60	70
RR	0.58	0.45-0.70	28	24	90
ROX	0.64	0.51-0.78	5,8	41	90
At 12 hours					
SpO_2/FiO_2	0.8	0.71-0.89	159	65	83
RR	0.72	0.61-0.83	25	60	77
ROX	0.78	0.67-0.89	5,99	64	96
At 24 hours					
SpO_2/FiO_2	0.7974	0.70-0.89	236	100	48
RR	0.7971	0.68-0.90	25.5	59	86
ROX	0.8258	0.73-0.91	8.36	86	66

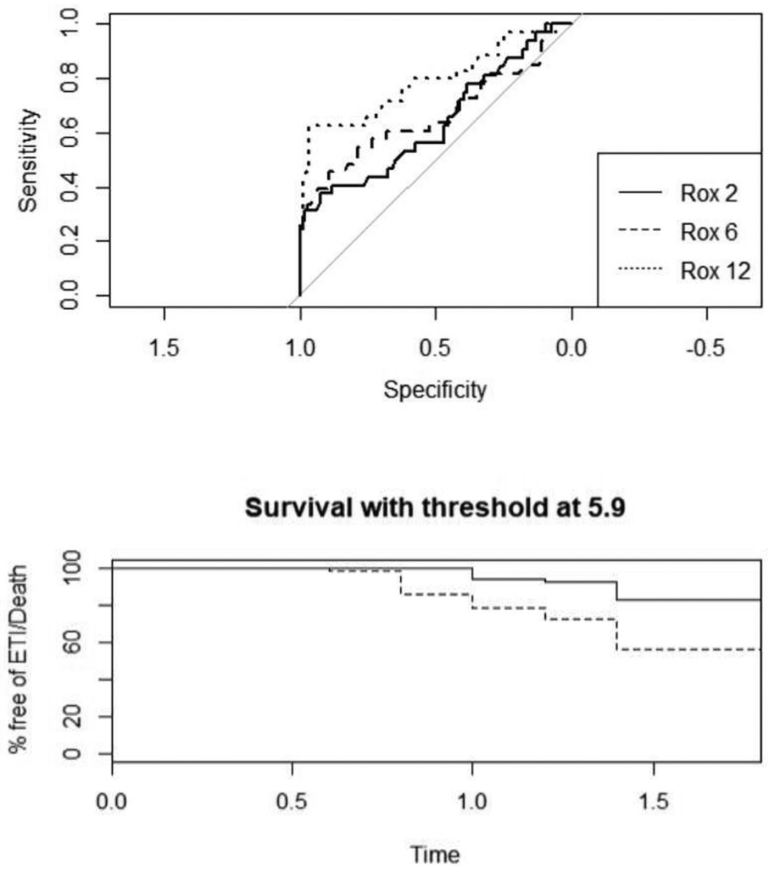

Fig. 1 Upper part: the ROC analysis identified the 12-hour ROX index as the best predictor of intubation with an AUC of 0.7916[CI 95% 0.6905-0.8927] compared to 2 and 6-hour ROX index. Best threshold to be 5.99[Specificity 96% Sensitivity 62%]. Lower part: In the survival analysis, a ROX value <5.99 was associated with an increased risk of failure (p = 0008 log − rank test).

Conclusion

In summary, this multicenter study provides evidence that the ROX index may be useful in guiding clinicians in their decision to intubate patients, especially patients with moderate ARF, treated therefore outside the ICU. Indeed, it also demonstrates a different threshold value than that reported for non-COVID patients, possibly related to the different mechanisms of hypoxia.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.pulmoe.2021.04.003.

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ORIGINAL ARTICLE

Practice of tracheostomy in patients with acute respiratory failure related to COVID—19 — Insights from the PRoVENT—COVID study

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KEYWORDS
Ventilation management;
Tracheostomy;
Acute respiratory failure;
ARDS;
Covid-19;
Coronavirus 2019;
SARS-COV-2

Abstract
Objective: Invasively ventilated patients with acute respiratory failure related to coronavirus disease 2019 (COVID—19) potentially benefit from tracheostomy. The aim of this study was to determine the practice of tracheostomy during the first wave of the pandemic in 2020 in the Netherlands, to ascertain whether timing of tracheostomy had an association with outcome, and to identify factors that had an association with timing.
Methods: Secondary analysis of the 'PRactice of VENTilation in COVID—19' (PRoVENT—COVID) study, a multicenter observational study, conducted from March 1, 2020 through June 1, 2020 in 22 Dutch intensive care units (ICU) in the Netherlands. The primary endpoint was the proportion of patients receiving tracheostomy; secondary endpoints were timing of tracheostomy, duration of ventilation, length of stay in ICU and hospital, mortality, and factors associated with timing.
Results: Of 1023 patients, 189 patients (18.5%) received a tracheostomy at median 21 [17 to 28] days from start of ventilation. Timing was similar before and after online publication of an amendment to the Dutch national guidelines on tracheostomy focusing on COVID—19 patients (21 [17—28] vs. 21 [17—26] days). Tracheostomy performed $\leq$ 21 days was independently associated with shorter duration of ventilation (median 26 [21 to 32] vs. 40 [34 to 47] days) and higher mortality in ICU (22.1% vs. 10.2%), hospital (26.1% vs. 11.9%) and at day 90 (27.6% vs. 14.6%). There were no patient demographics or ventilation characteristics that had an association with timing of tracheostomy.
Conclusions: Tracheostomy was performed late in COVID—19 patients during the first wave of the pandemic in the Netherlands and timing of tracheostomy possibly had an association with outcome. However, prospective studies are needed to further explore these associations. It remains unknown which factors influenced timing of tracheostomy in COVID—19 patients.

Introduction

Tracheostomy is a frequently performed intervention in critically ill patients who require prolonged invasive ventilation, facilitating liberation from the ventilator and possibly reducing sedation needs due to increased patient comfort.[1] Patients with acute respiratory failure related to coronavirus disease 2019 (COVID—19) often need prolonged ventilation[2-4] and therefore, a substantial proportion of COVID—19 patients might benefit from tracheostomy.[5]

It is uncertain how long after start of invasive ventilation tracheostomy should be performed, in both non COVID—19 [6-8] and COVID—19 patients. Early in the pandemic, adjusted tracheostomy guidelines for COVID—19 patients were released worldwide,[9,10] with over 90% of these adjusted guidelines advising to wait at least 14 days after intubation before performing a tracheostomy.[10] Factors in favor of delaying tracheostomy included an expected decrease in risk of contamination of healthcare workers, and high risk of dangerous deteriorations in gas exchange during the procedure.[11] Timing could also have been influenced by shortages in personal protection equipment (PPE) and tracheostomy kits, and lack of experienced health care workers able to perform or guide the procedure.

The practice of tracheostomy in invasively ventilated patients with acute respiratory failure related to COVID—19 in the Netherlands is largely unknown. We explored the database of the 'PRactice of VENTilation in COVID—19' (PRoVENT—COVID) study, which contains epidemiological characteristics, granular ventilation data, and outcomes of more than 40% of all COVID—19 patients who needed invasive ventilation during the first 3 months of the outbreak in the Netherlands.[12] The aim of this study was to determine the practice of tracheostomy and to ascertain whether timing of tracheostomy had an association with duration of ventilation, length of stay and mortality. We also aimed to identify factors that had an association with timing of tracheostomy.

Methods

Study design, patients and data collection

This is a secondary analysis of the PRoVENT—COVID study (**eMethods** in **Online Supplement**). Patients were enrolled if: (1) aged ≥18 years; (2) admitted to one of the participating ICUs; and (3) had received invasive ventilation for respiratory failure related to COVID—19. Patients with unknown tracheostomy status due to transfer to a non—participating hospital, were excluded from this analysis.

Baseline characteristics were collected at start of invasive ventilation or ICU admission. Detailed variables and parameters of ventilation management were collected over the first 4 calendar days thereafter. ICU complications, including thromboembolic events, acute kidney injury and use of renal replacement therapy (RRT) were collected until day 28, as was reintubation status. Admission status and life status were collected until day 90.

Endpoints

The primary endpoint of this analysis was incidence of tracheostomy. Secondary endpoints were timing of

tracheostomy (counted as the number of days between start of invasive ventilation and tracheostomy procedure), outcomes including duration of ventilation, ICU− and hospital−length of stay (LOS), and ICU−, hospital−, and day 28− and day 90−mortality, and factors associated with timing.

National Dutch guidelines on tracheostomy

Before the pandemic, Dutch guidelines regarding tracheostomy practice in invasively ventilated ICU patients advised to 'consider a tracheostomy as soon as it is apparent that weaning from artificial ventilation is unlikely to happen within 2 weeks after endotracheal intubation', and also 'because this prediction is difficult, it is advised to generally delay the procedure until at least 10 days after intubation'.[13] The amendment focusing on tracheostomy in COVID−19 patients was released on April 23, 2020[14] and advised to wait at least 2 weeks before performing tracheostomy and if possible, to further delay the procedure to reduce the risk of viral transmission.

Statistical analysis

Continuous variables are presented as medians with interquartile ranges, and categorical variables as number and percentages. Timing of tracheostomy is shown in cumulative distribution plots stratified by (1) month of admission; (2) the median timing of tracheostomy of this cohort; and (3) admission before and after introduction of the amended national guideline.

Baseline characteristics and outcomes were compared between groups defined by tracheostomy status and by the median timing of this cohort. Wilcoxon rank−sum test for continuous variables and Fisher exact test for categorical variables were used accordingly. Duration of ventilation was compared through a clustered Fine−Gray competing risk model, with death before extubation treated as competing risk. Both survivors and non−survivors were included in this analysis. Binary outcomes were compared with adjusted odds ratios from a mixed−effect generalized linear model with a binomial distribution. Twenty−eight and 90−day mortality and ICU− and hospital−LOS were compared with adjusted hazard ratios from a Cox proportional hazard model. All models were adjusted for variables with a known or suspected relationship with outcome in COVID−19 patients, including age, gender, body mass index (BMI), partial arterial oxygen pressure to oxygen fraction (PaO_2/FiO_2) and plasma creatinine level at baseline, hypertension, diabetes mellitus, use of angiotensin converting enzyme inhibitors, use of angiotensin II receptor blockers, use of inotrope or vasopressor at start of ventilation, fluid balance, arterial pH, mean arterial pressure, heart rate, and respiratory system compliance at start of ventilation. The center and week of admission were considered as random effect. The group receiving tracheostomy and the group with timing greater than the median were used as references. In addition, duration of ventilation, LOS in survivors and 90−day mortality were compared using Kaplan−Meier estimators.

To ascertain which clinical factors had an independent association with timing of tracheostomy, a mixed−effect generalized linear model with Gaussian distribution and with center as random effect was used and reported as mean difference and 95% confidence interval (CI). The following variables with a known or suspected relationship with timing of tracheostomy were selected: 1) week of admission in participating hospital, the first week being determined by the date of the first COVID−19 admission in the ICU of that particular hospital; 2) demographic characteristics (age, gender, BMI, diabetes, hypertension, heart failure, asthma, and obstructive sleep apnea syndrome); 3) ventilatory and oxygenation variables in the first day after intubation or admission, aggregated as the median from a maximum of six assessments (tidal volume, positive end−expiratory pressure, respiratory system compliance, and PaO_2/FiO_2); 4) laboratory tests and vital signs in the first day after intubation or admission, aggregated as the median from a maximum of six assessments (arterial pH, lactate, creatinine, heart rate and mean arterial pressure); 5) organ support during the first day after intubation or admission (use of vasopressors, use of neuromuscular blocking agents (NMBA) and fluid balance); 6) use of prone positioning in the first 4 days of ventilation; 7) incidence of complications (thromboembolic events, acute kidney injury, use of RRT); 8) need of reintubation; and 9) being admitted after the online publication of the COVID−19 amendment to the Dutch national guidelines for tracheostomy. Missing data in predictors, when present in less than 5% of the patients, were imputed by the median.

In a post hoc analysis, we also examined whether the abovementioned variables were independently associated with performance of tracheostomy.

All analyses were conducted in R v.4.0.2 (R Foundation, Vienna, Austria) and significance level was set at 0.05.

Results

Patients

Between March 1 and June 1, 2020, 31 ICUs were invited for participation in the PRoVENT−COVID study and 22 met inclusion criteria. Of 1340 screened patients, 1023 were included in the current analysis (eFig. 1); the main reasons for exclusion were not having received invasive ventilation, unknown tracheostomy status due to transfer to a non−participating hospital, or having received ventilation for something other than COVID−19.

Incidence of tracheostomy

Of 1023 patients, 189 patients (18.5%) underwent tracheostomy. Tracheostomized patients were more often males and more likely to have asthma. On the first day of ventilation, tracheostomized patients had a higher respiratory compliance and $etCO_2$, and underwent longer duration of prone positioning (eTable 1). Performance of tracheostomy was independently associated with reintubation, pulmonary embolism, AKI and use of RRT. Additionally, tracheostomy was independently associated with longer duration of ventilation and lower ICU−, hospital−, and 28− and 90−day mortality (eTable 2).

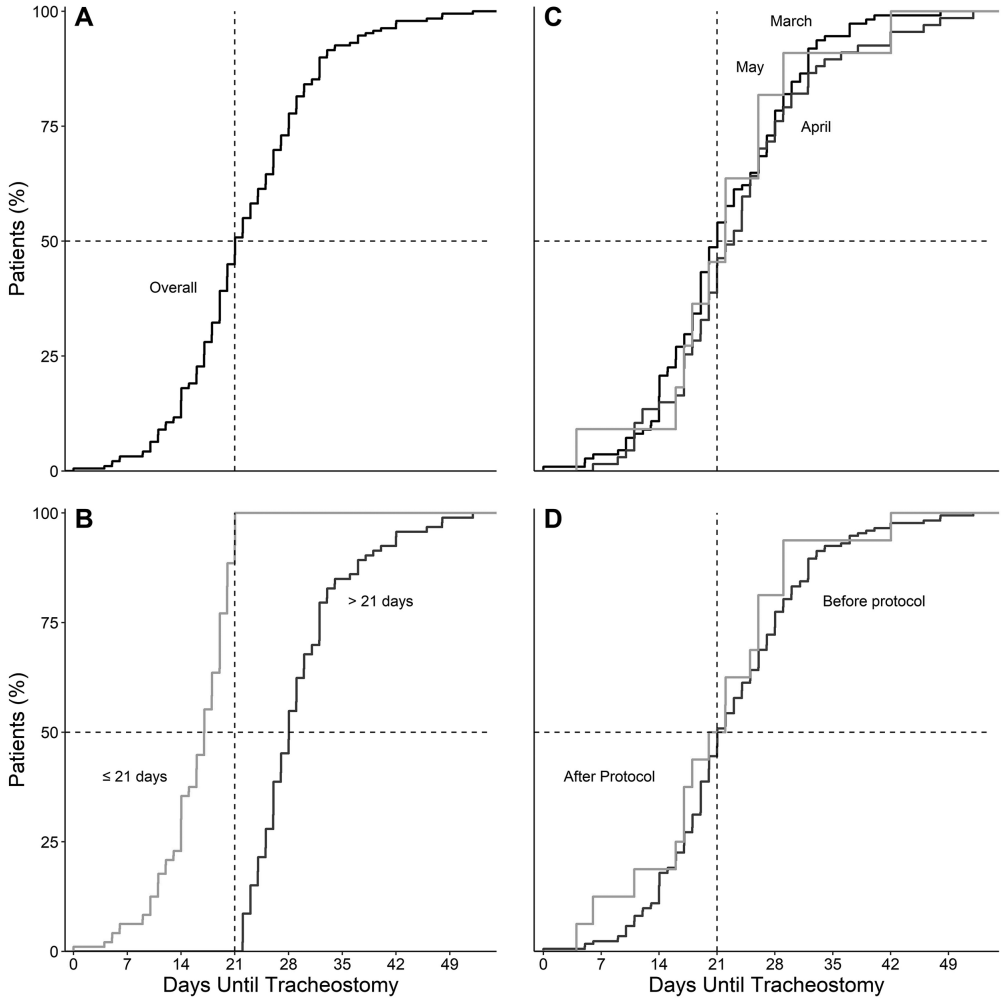

Figure 1 Cumulative Distribution Curves of Timing of Tracheostomy (A): All tracheostomized patients; (B): Stratified by month of admission; (C): Stratified by timing of tracheostomy before or on the median, or after; (D): Stratified by admission before and after the protocol.

Timing of tracheostomy

Time between start of invasive ventilation and tracheostomy was median 21 [17—28] days. Time until tracheostomy in March (21 [16—28] days), April (23 [17—28] days) and May (22 [17—26] days) was similar and seemed unaffected by the online publication of the amendment (21 [17—28] vs. 21 [17—26] days) (Fig. 1).

Compared to patients who were tracheostomized $>$ 21 days, patients who were tracheostomized $\leq$ 21 days had a lower plasma creatinine and higher respiratory compliance and were ventilated with a higher tidal volume at baseline (Table 1). Tracheostomy $\leq$ 21 days was independently associated with shorter duration of ventilation, but higher ICU—, hospital— and 90—day mortality (Table 2 and Fig. 2).

Factors associated with timing and performance of tracheostomy

None of the clinical factors were associated with timing of tracheostomy (eTable 3). In the post hoc analysis, independent predictors of performance of tracheostomy were asthma, respiratory system compliance, occurrence of thromboembolic complications, need for RRT and reintubation (eTable 4).

Discussion

The findings of this secondary analysis of the PRoVENT—COVID study can be summarized as follows: (1) roughly 1 in every 5 patients were tracheostomized, (2) median timing of tracheostomy was 21 days, and this remained after online publication of the amendment of the national guideline, (3) tracheostomy $\leq$ 21 days had an independent association with shorter duration of ventilation and higher mortality rates, and (4) timing was not influenced by clinical factors. In a post hoc analysis, asthma, respiratory system compliance, occurrence of thromboembolic complications, need for RRT and reintubation were associated with performance of tracheostomy.

This analysis has several strengths. First, it is one of the largest cohorts of COVID—19 patients in which tracheostomy practice was analyzed. Second, we used data captured over the entire first wave of the pandemic in the Netherlands, encompassing about 40% of all ICU patients admitted during

Table 1 Baseline characteristics of patients receiving tracheostomy according to timing.

	Timing of tracheostomy[a]		p value
	≤ 21 days (*n* = 96)	> 21 days (*n* = 93)	
Age, years	65.0 (59.0 - 72.0)	65.0 (60.0 - 71.0)	0.947
Male gender — no (%)	75 (78.1)	77 (82.8)	0.466
Body mass index, kg/m^2	26.5 (24.7 - 31.0)	28.0 (26.1 - 29.9)	0.265
Transferred under invasive ventilation	21 (21.9)	17 (18.3)	0.589
Days between intubation and admission	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	0.922
Use of non-invasive ventilation — no (%)	4 / 79 (5.1)	7 / 86 (8.1)	0.539
Duration of non-invasive ventilation, hours	8.0 (5.0 - 36.0)	4.5 (1.0 - 14.8)	0.356
Timing of tracheostomy, days	17.0 (14.0 - 19.0)	28.0 (25.0 - 32.0)	< 0.001
Admitted after the publication of guideline[b]	8 (8.3)	8 (8.6)	0.999
Week of admission within the center[c]	2.5 (1.0 - 4.0)	3.0 (2.0 - 4.0)	0.269
Chest CT scan performed — no (%)	26 / 91 (28.6)	32 / 89 (36.0)	0.339
Lung parenchyma affected — no (%)			0.681
25%	11 / 26 (42.3)	9 / 32 (28.1)	
50%	9 / 26 (34.6)	12 / 32 (37.5)	
75%	5 / 26 (19.2)	9 / 32 (28.1)	
100%	1 / 26 (3.8)	2 / 32 (6.2)	
Chest X-ray performed — no (%)	55 / 63 (87.3)	49 / 55 (89.1)	0.999
Quadrants affected — no (%)			0.583
1	5 / 55 (9.1)	8 / 50 (16.0)	
2	14 / 55 (25.5)	15 / 50 (30.0)	
3	17 / 55 (30.9)	11 / 50 (22.0)	
4	19 / 55 (34.5)	16 / 50 (32.0)	
Severity of ARDS — no (%)			0.198
Mild	18 (18.8)	21 / 90 (23.3)	
Moderate	74 (77.1)	60 / 90 (66.7)	
Severe	4 (4.2)	9 / 90 (10.0)	
Co-existing disorders — no (%)			
Hypertension	29 (30.2)	33 (35.5)	0.536
Heart failure	4 (4.2)	6 (6.5)	0.532
Diabetes	22 (22.9)	21 (22.6)	0.999
Chronic kidney disease	6 (6.2)	5 (5.4)	0.999
Baseline creatinine, μmol/L[d]	74.0 (65.0 - 85.5)	81.0 (65.0 - 105.0)	0.032
Liver cirrhosis	1 (1.0)	0 (0.0)	0.999
Chronic obstructive pulmonary disease	6 (6.2)	8 (8.6)	0.588
Active hematological neoplasia	1 (1.0)	3 (3.2)	0.363
Active solid neoplasia	3 (3.1)	2 (2.2)	0.999
Neuromuscular disease	0 (0.0)	1 (1.1)	0.492
Immunosuppression	4 (4.2)	1 (1.1)	0.369
Asthma	10 (10.4)	9 (9.7)	0.999
Obstructive sleep apnea syndrome	5 (5.2)	5 (5.4)	0.999
Previous medication — no (%)			
Systemic steroids	5 (5.2)	2 (2.2)	0.445
Inhalation steroids	11 (11.5)	9 (9.7)	0.814
Angiotensin converting enzyme inhibitor	17 (17.7)	16 (17.2)	0.999
Angiotensin II receptor blocker	9 (9.4)	14 (15.1)	0.270
Beta-blockers	22 (22.9)	18 (19.4)	0.596
Insulin	9 (9.4)	5 (5.4)	0.407
Metformin	13 (13.5)	14 (15.1)	0.837
Statins	32 (33.3)	28 (30.1)	0.643
Calcium channel blockers	13 (13.5)	15 (16.1)	0.685
Organ support at start of ventilation — no (%)			
Continuous sedation	92 (95.8)	87 (93.5)	0.532
Inotropic or vasopressor	77 (80.2)	66 (71.0)	0.175
Vasopressor	77 (80.2)	66 (71.0)	0.175
Inotropic	1 (1.0)	5 (5.4)	0.114
Fluid balance, mL	585.5 (43.5 - 1438.6)	357.9 (-61.5 - 965.5)	0.138
Urine output, mL	682.5 (347.5 - 1172.5)	762.5 (411.2 - 1171.2)	0.579

Table 1 (*Continued*)

	Timing of tracheostomy[a]		p value
	≤ 21 days (*n* = 96)	> 21 days (*n* = 93)	
Ventilation support at start of ventilation			
Assisted ventilation — no (%)	29 (30.2)	23 / 92 (25.0)	0.514
Tidal volume, mL/kg PBW	6.7 (6.1 - 7.6)	6.4 (5.8 - 6.9)	0.019
PEEP, cmH_2O	13.0 (11.2 - 14.6)	12.5 (10.7 - 14.3)	0.478
Peak pressure, cmH_2O	26.0 (23.4 - 29.1)	25.7 (23.9 - 29.0)	0.734
Driving pressure, cmH_2O	13.0 (11.0 - 15.1)	14.0 (12.0 - 15.9)	0.090
Mechanical power, J/min	18.9 (15.5 - 22.7)	19.1 (15.7 - 23.2)	0.882
Compliance, mL/cmH_2O	37.7 (30.9 - 45.3)	33.5 (26.4 - 40.1)	0.037
Total respiratory rate, mpm	21.2 (19.0 - 24.0)	22.3 (19.4 - 24.5)	0.352
FiO_2	0.60 (0.49 - 0.69)	0.56 (0.47 - 0.66)	0.344
$etCO_2$, mmHg	36.9 (33.3 - 41.6)	38.4 (34.2 - 44.8)	0.290
Vital signs at start of ventilation			
Heart rate, bpm	88.5 (76.4 - 101.1)	84.0 (77.0 - 100.2)	0.429
Mean arterial pressure, mmHg	80.3 (73.5 - 87.5)	79.9 (75.3 - 91.3)	0.484
Laboratory tests at start of ventilation			
pH	7.36 (7.30 - 7.40)	7.36 (7.31 - 7.41)	0.473
PaO_2, mmHg	81.4 (74.1 - 94.3)	81.8 (73.1 - 98.0)	0.896
PaO_2 / FiO_2, mmHg	123.9 (93.4 - 160.8)	140.6 (109.0 - 200.7)	0.074
$PaCO_2$, mmHg	45.5 (40.3 - 51.9)	45.0 (39.8 - 50.5)	0.553
Lactate, mmol/L	1.1 (0.9 - 1.5)	1.1 (1.0 - 1.4)	0.555
Adjunctive therapies at start of ventilation			
Prone positioning - no. (%)	30 / 93 (32.3)	303 / 92 (32.6)	0.999
Duration of prone positioning, hours	9.5 (6.0 - 14.4)	10.0 (7.0 - 13.5)	0.897
Recruitment maneuvers - no. (%)	1 / 84 (1.2)	33 / 80 (3.8)	0.358
ECMO - no. (%)	0 / 95 (0.0)	0 (0.0)	—
Use of NMBA - no. (%)	29 (30.2)	20 (21.5)	0.188
Duration of neuromuscular blocking agents, hours	0.0 (0.0 - 8.0)	0.0 (0.0 - 0.0)	0.200

Data are median (quartile 25% - quartile 75%) or No (%). Percentages may not total 100 because of rounding
CT: computed tomography; ARDS: Acute Respiratory Distress Syndrome; PBW: predicted body weight; PEEP: positive end expiratory pressure; ECMO: extracorporeal membrane oxygenation; NMBA: neuromuscular blocking agent

[a] Groups defined by the median timing of this cohort.
[b] National guideline on practice of tracheostomy on COVID-19 patients published on April 23, 2020.
[c] First week determined as the week when the first patient was admitted in the center.
[d] Most recent measurement in 24 hours before intubation, or at ICU admission under invasive ventilation.

that time. Third, university, non—university, teaching and non—teaching centers were involved, increasing the generalizability of this study. Fourth, hospitals that were invited but did not participate were unable to do so only because of administrative and regulatory barriers, unrelated to the workload and possible lack of time on the ICU. This makes selection bias unlikely. Fifth, the exact date of publication of the tracheostomy guideline amendment was known, giving accurate insight into its influence on practice of tracheostomy. Sixth, our statistical analysis plan was published before analysis of the data, preventing reporting bias.

The incidence of tracheostomy in the current cohort of COVID—19 patients is slightly higher than in LUNG SAFE,[15] a large service review in which 13% of ARDS patients received a tracheostomy.[16] LUNG SAFE patients were ventilated for a shorter duration and represent a more heterogeneous population of ARDS patients, possibly explaining this difference. Also, thromboembolic complications and AKI are prevalent among COVID—19 ARDS patients,[17,18] factors which were shown to be predictors of performing a tracheostomy in this study.

The timing of tracheostomy was substantially later in this study than in LUNG SAFE, which showed a median timing of 14 days.[16] This may be due to the larger proportion of patients with moderate—severe ARDS seen in this study, as well as lower PaO_2/FiO_2 and use of higher PEEP at baseline. Placement of a tracheostomy canula can be unsafe in patients with severely compromised gas—exchange because of the unavoidable loss in positive airway pressure, delaying the procedure until improvement of oxygenation is seen. In addition, tracheostomy could have been postponed by health care providers out of fear of viral transmission, preferably waiting for a decrease in viral load.

Practice of tracheostomy is difficult to compare to other cohorts of invasively ventilated COVID—19 patients because of the large variability seen in both incidence and timing in a number of identified studies. Incidence of tracheostomy in

Table 2 Clinical outcomes of patients receiving tracheostomy according to timing.

	Timing of tracheostomy[a]		Adjusted effect estimate* (95% Confidence Interval)	*p* value
	≤ 21 days (*n* = 96)	> 21 days (*n* = 93)		
Duration of ventilation, days	26.0 (21.0 - 32.0)	40.0 (33.5 - 46.5)	SHR, 13.74 (5.48 to 34.47)[c]	< 0.001
In survivors at day 28, days	26.5 (21.8 - 33.3)	40.0 (33.5 - 46.5)		
Reintubation — no (%)	24 / 95 (25.3)	24 (25.8)	OR, 0.96 (0.46 to 1.99)[d]	0.902
Thromboembolic complications — no (%)	38 (39.6)	45 (48.4)	OR, 0.65 (0.35 to 1.22)[d]	0.181
Pulmonary embolism	32 (33.3)	38 (40.9)	OR, 0.70 (0.37 to 1.33)[d]	0.279
Deep vein thrombosis	3 (3.1)	9 (9.7)	OR, 0.26 (0.01 to 4.95)[d]	0.369
Ischemic stroke	3 (3.1)	5 (5.4)	OR, 0.13 (0.01 to 2.07)[d]	0.150
Myocardial infarction	2 (2.1)	1 (1.1)	—	—
Systemic arterial embolism	0 (0.0)	0 (0.0)	—	—
Acute kidney injury — no (%)	53 / 94 (56.4)	53 (57.0)	OR, 1.24 (0.58 to 2.62)[d]	0.578
Need for RRT — no (%)	26 (27.1)	35 (37.6)	OR, 0.59 (0.27 to 1.30)[d]	0.192
Need of rescue therapy — no (%)[b]	71 / 95 (74.7)	72 (77.4)	OR, 0.65 (0.29 to 1.49)[d]	0.312
Prone positioning	51 / 95 (53.7)	61 / 92 (66.3)	OR, 0.43 (0.18 to 1.04)[d]	0.060
Recruitment maneuver	5 / 85 (5.9)	6 / 82 (7.3)	OR, 0.73 (0.14 to 3.91)[d]	0.715
Use of NMBA	53 (55.2)	46 (49.5)	OR, 1.05 (0.50 to 2.19)[d]	0.894
ECMO	1 / 95 (1.1)	0 (0.0)	—	—
Use of continuous sedation — no (%)[b]	95 (99.0)	90 (96.8)	OR, ∞ (0.00 to ∞)[d]	0.999
Use of inotropic or vasopressor — no (%)[b]	90 (93.8)	85 (91.4)	OR, 0.00 (0.00 to ∞)[d]	0.407
Use of vasopressor	90 (93.8)	85 (91.4)	OR, 0.00 (0.00 to ∞)[d]	0.407
Use of inotropic	5 (5.2)	8 (8.6)	OR, 0.09 (0.00 to 2.67)[d]	0.166
ICU length of stay, days	29.0 (24.3 - 36.8)	44.0 (37.5 - 51.0)	HR, 1.18 (0.82 to 1.69)[e]	0.370
In survivors, days	29.0 (25.0 - 38.0)	43.5 (37.0 - 50.0)		
Hospital length of stay, days	39.0 (30.0 - 50.0)	58.0 (50.0 - 66.0)	HR, 1.05 (0.72 to 1.52)[e]	0.810
In survivors, days	43.0 (33.5 - 53.5)	58.0 (52.0 - 65.8)		
ICU mortality — no (%)	21 / 95 (22.1)	9 / 88 (10.2)	OR, 3.24 (1.24 to 8.46)[d]	0.016
Hospital mortality — no (%)	24 / 92 (26.1)	10 / 84 (11.9)	OR, 3.76 (1.44 to 9.85)[d]	0.007
28-day mortality — no (%)	11 (11.5)	0 (0.0)	—	—
90-day mortality — no (%)	24 / 87 (27.6)	12 / 82 (14.6)	HR, 3.24 (1.45 to 7.25)[e]	0.004

Data are median (quartile 25% - quartile 75%) or No (%). Percentages may not total 100 because of rounding
RRT: renal replacement therapy; NMBA: neuromuscular blocking agent; ECMO: extracorporeal membrane oxygenation; ICU: intensive care unit

* All models adjusted for age, gender, body mass index, PaO_2 / FiO_2, creatinine, hypertension, diabetes mellitus, use of angiotensin converting enzyme inhibitors, use of angiotensin II receptor blockers, use of inotrope or vasopressor at start of ventilation, fluid balance, pH, mean arterial pressure, heart rate, and respiratory system compliance at start of ventilation.

[a] Groups defined by the median timing of this cohort; group tracheostomized after 21 days is the reference.

[b] Assessed in the first four days of ventilation.

[c] Subdistribution hazard ratio from a Fine-Gray competing risk model with death before extubation in 28 days treated as a competing risk and with center as clustering effect.

[d] Odds ratio from a mixed-effect generalized linear model with a binomial distribution and with center as random effect.

[e] Hazard ratio from a (shared-frailty) Cox proportional hazard model (for the ICU and hospital length of stay analyses, all patients who died prior to discharge were assigned the maximum length of stay to account for death as a competing risk in this model) with center as frailty. P value for the Schoenfeld residuals; < 0.001 (ICU length of stay); < 0.001 (hospital length of stay); < 0.001 (90-day mortality)

invasively ventilated COVID−19 patients has been shown to range from 8 to 77%,[3,19-35] and mean or median timing from 4 to 23 days.[5,19,22,24,27-42] As many of these identified studies are single center, this may reflect differences in local practices irrespective of national guidelines. This idea is supported by the fact that most of the identified studies showed a timing of ≤ 14 days,[19,22,24,27,28,32,33,35,39,42] despite 90% of international guidelines recommending waiting at least 14 days before performing a tracheostomy in COVID−19 patients.[10] The timing in our cohort, however, is in line with the majority of international guidelines, as well as the amendment of national Dutch tracheostomy guidelines. Therefore, it is possible that international advice regarding tracheostomy practice, released early on in the pandemic of 2020, was followed even before publication of the amendment of national guidelines. As it was already national practice to delay tracheostomy until at least 10 days after intubation, it would not have been a significant change in mindset.

Earlier timing of tracheostomy had an association with shorter duration of ventilation in this cohort. Freeing patients from the ventilator as soon as possible is particularly valuable when there is a surge of patients needing to be treated in a pandemic. However, earlier tracheostomy was also associated with a higher mortality. This association was found after correcting for factors possibly having an

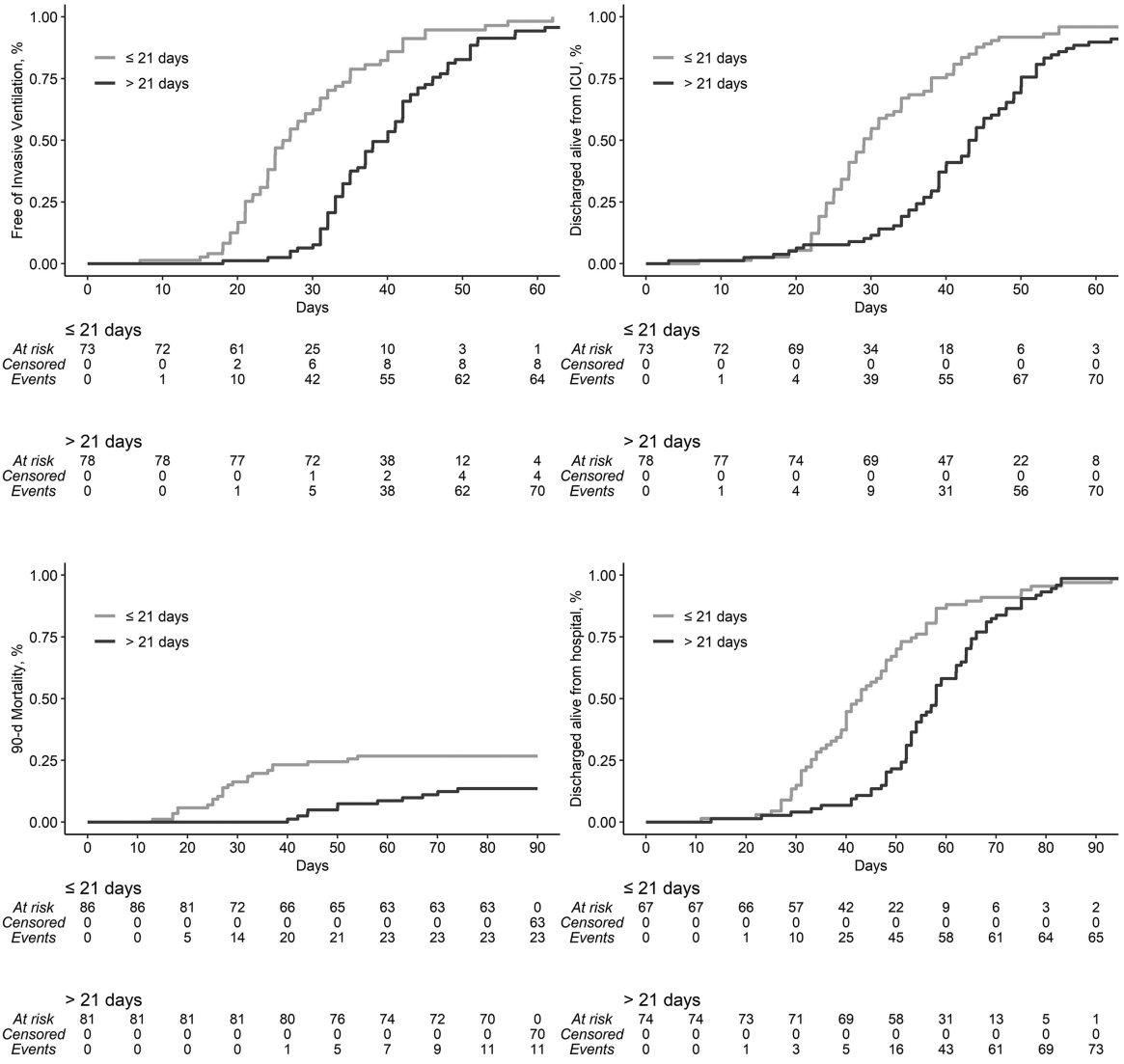

Figure 2 Kaplan—Meier Curves stratified by Timing of Tracheostomy (A): Duration of Ventilation in Survivors; (B): 90—day survival; (C): Duration of ICU stay in survivors; (D): Duration of hospital stay in survivors

independent association with outcome, such as hemodynamic and respiratory problems. It is likely that this association is the result of immortal time bias and not an actual cause and effect. This phenomenon is introduced when patients cannot experience a certain outcome during a period of follow—up. In this analysis, patients in the later tracheostomy group already had survived 21 days before being categorized. This period is quite long, which may have increased the magnitude of bias.[43]

This study has limitations. We had 99 patients with an unknown tracheostomy status due to transfer to a non—participating hospital. In addition, we did not collect local guidelines on tracheostomy practice, making it unclear what decisions were precisely based on. Different approaches to clinical decisions from each center may have influenced outcomes. We didn't collect data on PPE and tracheostomy kit shortages and cannot determine the effect this had on timing. Whether a surgical or percutaneous technique was used for placement of tracheostomy was not recorded, which could have given additional insight into tracheostomy practice in the Netherlands. Ventilation data was restricted to the first four days of admission and therefore are unknown directly before placement of tracheostomy. Furthermore, data regarding incidence of ventilator associated pneumonia (VAP), other infections and sepsis were not collected; as these factors could influence both timing of tracheostomy and outcome, this is a limitation. Finally, use of additional therapies such as antibiotics, steroids and anticoagulants is unknown in our cohort, which could also have influenced outcome.

Conclusion

This study gives insight into the practice of tracheostomy in invasively ventilated COVID—19 patients in the Netherlands. The results suggest that earlier timing of tracheostomy is associated with a shorter duration of ventilation, which would be of crucial benefit in overloaded ICUs. However, because this study also suggested a higher mortality in patients who received earlier tracheostomy, future studies are needed to determine whether earlier tracheostomy has a positive influence on outcome in COVID—19 patients. It

remains unclear which factors influenced timing of tracheostomy during the first wave of the pandemic.

Conflicts of interest

The authors have no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.pulmoe.2021.08.012.

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ORIGINAL ARTICLE

Molecular analysis in cytological samples obtained by endobronchial or oesophageal ultrasound guided needle aspiration in non-small cell lung cancer

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PEN membrane slide

Abstract
Introduction: Cytological samples obtained by endobronchial ultrasound (EBUS) are capital for diagnosis, staging and molecular profile in non-small cell lung carcinoma (NSCLC).
Objective: To assess the success rate of complete, partial and individual of molecular analysis in samples obtained by EBUS-guided transbronchial needle aspiration (TBNA) and/or by oesophageal ultrasound-guided fine needle aspiration with an echobronchoscope (EUS-B-FNA) in patients with NSCLC.
Methods: Prospective study including 90 patients with non-squamous NSCLC, or non-smoking squamous. Cytological samples were classified into two groups. Group 1: PEN membrane slide and/or cell blocks for the determination of mutations of EGFR, KRAS, ERBB2 and BRAF. Group 2: silane coated slides or cell blocks for rearrangements of ALK, ROS1 and MET amplification.
Results: The success rate was 78.6% for 4 molecular alterations (EGFR, KRAS, ALK and ROS1), and 44% for 7 determinations. The individual success rate for EGFR was 97%, KRAS 96.3%, ALK 85%, ROS1 82.3%, ERBB2 71.4%, BRAF 67.7% and MET 81.1%. There were no significant differences ($p = 0.489$) in the number of molecular analyses (1–3 vs. 4) in group 1, depending on the types of samples (cell block vs. PEN membrane slide vs. cell block and PEN membrane slide).

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Conclusions: In patients with NSCLC, the cytological material obtained by ultrasound-guided needle aspiration is sufficient for individual and partial molecular analysis in the vast majority of cases. Membrane slides such as cell blocks are valid samples for molecular analysis.

Introduction

Lung cancer is the most frequent malignant neoplasm and the main cause of cancer mortality.[1] NSCLC accounts for 85% of all lung cancers, with adenocarcinoma as the most prevalent histological subtype.[1,2] Currently, the identification of different oncogenic alterations leads to oncospecific treatment which improves the overall response rate and progression-free survival in this group of patients.[3,4]

The diagnostic and/or staging process includes different minimally invasive and invasive techniques to obtain cytological and/or histological material, which provides differentiation of the histological subtype and identification of molecular markers according to treatment guidelines.[5,6] EBUS-TBNA is a cost-effective tool for obtaining cytological samples safely, less invasively, and with a lower complication rate, when compared to surgical techniques.[7,8] Several studies have shown that the performance of isolated molecular determinations is feasible in 70–90% of samples obtained by EBUS-TBNA.[9,10] Nevertheless, for newer onco-targeted treatments, a greater amount of cyto/histological material is required in order to analyse a greater number of possible molecular targets or resistances.

The main objective of our study was to assess the success rate in the determination of molecular alterations (EGFR – epidermal growth factor receptor, KRAS – kirsten rat sarcoma viral oncogene homolog, ALK – anaplastic lymphoma receptor tyrosine kinase, ROS1 – proto-oncogene tyrosine-protein kinase ROS, ERBB2 – erb-b2 receptor tyrosine kinase 2, BRAF – v-Raf murine sarcoma oncogene homolog B1 and MET – tyrosine-protein kinase Met) in a complete, partial and individual way, from samples obtained by EBUS-TBNA and/or EUS-B-FNA in patients with NSCLC. The secondary objectives of this study were to study its prevalence in our population, and to compare the usefulness of different cytological samples for mutation analysis.

Methods

Patients

This is an observational prospective study conducted in a tertiary hospital in patients with suspected or known NSCLC undergoing EBUS-TBNA and/or EUS-B-FNA for diagnosis and/or staging study, from January 2013 to December 2016. The inclusion criteria were patients with advanced stages (IIIB, IV), or stage IIIA non-tributary of surgical treatment, and cases with suspected recurrence or disease progression, in the absence of an active treatment.

Current smokers with squamous carcinoma were excluded. Our study was approved by the Ethics Committee of our hospital (PI-14-073), and the patients signed informed consent forms.

Procedures

TBNA or FNA were performed under local anaesthesia with lidocaine, and with moderate sedation with midazolam, propofol and/or remifentanil. The convex probe EBUS (UC 180F, Olympus Optical Co Ltd., Tokyo, Japan) was used, and sampling was performed with the 22-gauge cytology needle (NA2015X-4022; Olympus Optical Co, Tokyo, Japan). Mediastinal and/or hilar nodes with a diameter of the minor axis $\geq$5 mm and central masses were punctured. The bronchoscopist proceeded from nodes in regions corresponding to N3 disease to regions of N1 disease.[8] We performed 1–7 punctures per node and/or mass with a maximum of 15 revolutions per puncture, and negative pressure was used ($-20\,cmH_2O$) in most cases.

Preparation of cytological samples

The aspirates obtained were placed on slides, fixed with 96% ethanol and stained with haematoxylin. Rapid on-site evaluation (ROSE) was performed by a cytopathologist. Aspirates were considered satisfactory with the following criteria: (1) Benign: 40 lymphocytes per high-power field in cellular areas of the smear and/or clusters of pigmented macrophages without evidence of malignant cells or (2) Metastatic: presence of malignant cells. Samples were considered inadequate if they showed only bronchial/oesophageal cells, erythrocytes or necrotic tissue.

From the metastatic aspirate, two cell blocks $\pm$ PEN (polyethylene naphthalate) membrane slide (Carl Zeiss, membrane slide NF 1.0 PEN, Germany) were prepared, and if it was not feasible (even with procoagulants), 4 extensions were performed on silane coated slides, and 1 PEN membrane slide. The cell blocks were prepared by air-drying the slides to clot, fixed in 10% formalin, and subsequently analysed in laboratory. Blocks were included in paraffin and sectioned (4 μm thickness, 8–10 slides), two for with haematoxylin–eosin staining, two slides for immunohistochemistry staining (IHC) such as thyroid transcription factor 1 (TTF-1) and/or p40, the remaining slides were later used for sequential molecular analysis (Fig. 1).

The samples obtained were divided into two groups. Group 1: PEN membrane slide and/or cell blocks and were used to perform PCR for molecular analysis of EGFR, KRAS, BRAF and ERBB2. Group 2: silane coated slide or cell blocks and were used to perform FISH techniques for ALK, ROS1, and MET studies.

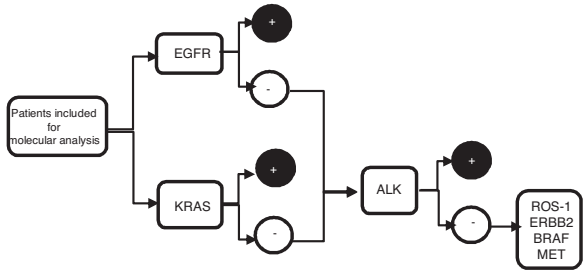

Figure 1 Algorithm of sequential molecular analysis. EGFR: epidermal growth factor receptor; KRAS: kirsten rat sarcoma viral oncogene homolog; ALK: anaplastic lymphoma receptor tyrosine kinase; ROS1: protooncogene tyrosine protein kinase ROS; ERBB2: erb-b2 receptor tyrosine kinase 2; BRAF: v-Raf murine sarcoma oncogene homolog B1; MET: tyrosine-protein kinase Met.

Laboratory methods

Detection of EGFR, KRAS, BRAF, ERBB2 genes mutations
The polymerase chain reaction (PCR) technique was used from cell blocks or membrane slides, where tumour cell extraction was performed using laser microdissection techniques (Carl Zeiss MicroImaging GmbH, München, Germany). DNA was extracted with phenol–chloroform–isoamyl alcohol. If tumour cells were <200, a lysis buffer with proteinase K compatible with the PCR buffer was used (Ecogen, Barcelona, Spain). For the determination of deletions of exon 19 of the EGFR, the GeneScan technique (Applera, Norwalk, CT, USA) was used, while for the detection of the L858R mutation of exon 21 and T790M of exon 20 the Taqman technique was used (Applied Biosystems).

Genomic *KRAS codons 12 and 13* mutations were assessed by the Sanger technique. Taqman technique was used for V600 mutations in BRAF and exon 20 in ERBB2.

Detection of ALK, ROS1 genes rearrangements and MET gene amplification
Fluorescence in situ hybridisation (FISH) was used with the following probes: the Vysis ALK Break-Apart (Abbott Molecular, Inc., Des Plaines, IL, USA) for ALK, ZytoLight® SPEC ROS1 Dual Color Break Apart (ZytoVision, Bremerhaven, Germany) for ROS1, and ZytoLight® MET/CEP 7 Dual Color (ZytoVision, Bremerhaven, Germany) for MET. After dewaxing, the slides were processed with the Histology FISH Accessory kit (Dako, Denmark) and hybridisation titration was carried out with an Olympus BX51 fluorescence microscope. A minimum of 50 tumour cells was necessary. For ALK and ROS1 genes rearrangements, samples were considered positive when $\geq$15% of the cells showed a positive hybridisation pattern. For the MET gen, the criteria described by Noonan et al.[11] was used according to a ratio of MET/centromere $\geq$1.8.

Statistical analysis

Categorical variables were expressed in relative and absolute frequencies; continuous variables in mean and standard deviation when they presented a normal distribution, or as median and interquartile range when they did not present a normal distribution.

Table 1 General characteristics of patients.

Baseline characteristics	Patients ($n = 90$)
Age	65.2 ± 9.4
Sex, n (%)	
Male	74 (82.2%)
Smoking history, n (%)	
Never-smoker	8 (9%)
Former smoker	35 (38.8%)
Current smoker	47 (52.2%)
Histological type, n (%)	
Adenocarcinoma	71 (78.9%)
Squamous carcinoma	3 (3.3%)
Carcinoma NOS	16 (17.8%)
Stages, n (%)	
Inoperable IIIA	31 (34.4%)
Multi-level N2	11
Persistent N2	5
Poor lung function	11
Performance status 3–4	4
IIIB	16 (17.8%)
IV	43 (47.8%)
Procedure	
EBUS-TBNA	76 (84.4%)
EUS-B-FNA	11 (12.2%)
Both	3 (3.3%)
Molecular study, n (%)	
Yes	87 (96.7%)
No	3 (3.3%)
Final treatment	
Oncospecific	11 (12.2%)
Conventional (chemotherapy and/or radiotherapy)	62 (68.9%)
	17 (18.9%)
None → (rapid → progression → or → poor → performance)	

Carcinoma NOS (not otherwise specified) type; EBUS-TBNA (endobronchial ultrasound guided biopsy); EUS-B-FNA (oesophageal ultrasound-guided fine needle aspiration with an echobronchoscope).

The success rate for the complete or individual molecular analysis in the samples obtained by EBUS-TBNA or EUS-B-FNA was calculated. The relationships between categorical variables were analysed using the chi-square test or Fisher's exact test. Values of $p < 0.05$ were considered statistically significant. Data were analysed using IBM SPSS software version 24.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

Ninety patients were included in the study. In 54 cases (60%), EBUS/EUS-B was the first diagnostic procedure performed. Seventy-one patients (78.9%) had a diagnosis of adenocarcinoma. Baseline characteristics are shown in Table 1.

Table 2 Individual molecular analysis.

Type of molecular study	Number of analysed samples	Enough (%)	Positive (%)	Negative (%)
EGFR	87	84 (97%)	13 (15%)	71 (82%)
KRAS	81	78 (96.3%)	18 (22.2%)	60 (74.1%)
ALK	80	68 (85%)	1 (1.3%)	67 (83.7%)
ROS1	79	65 (82.3%)	0	65 (82.3%)
ERBB2	63	45 (71.4%)	0	45 (71.4%)
BRAF	62	42 (67.7%)	0	42 (67.7%)
MET	37	30 (81.1%)	1 (2.7%)	29 (78.3%)

EGFR: epidermal growth factor receptor; KRAS: kirsten rat sarcoma viral oncogene homolog; ALK: anaplastic lymphoma receptor tyrosine kinase; ROS1: protooncogene tyrosine protein kinase ROS; ERBB2: erb-b2 receptor tyrosine kinase 2; BRAF: v-Raf murine sarcoma oncogene homolog B1; MET: tyrosine-protein kinase Met.

Of the 12 pulmonary lesions (24–63 mm of short-axis diameter) and 329 hilar and/or mediastinal lymphadenopathies punctured, 114 (30.6%) were malignant. The most frequently punctured metastatic lymph nodes stations were 7 (33), 4R (30), 4L (12) and 10/11/12 (11). The mean short-axis diameter of the lymph node was 13.3 ± 6.8 mm and the average number of punctures per node was 3 (range 1–7).

Histological subclassification

In 35 cases (39%), the final diagnosis was obtained by morphological assessment, while IHC (TTF-1, p40) was used in 53 (58.9%). A definitive subtyping (morphology and/or IHC) was achieved in 82.2% vs. 17.8% of carcinoma NOS (not otherwise specified). In this subgroup of 14 cases, both morphological assessment and IHC were inconclusive, while in 2 cases IHC was not performed because the material for the molecular analysis was prioritised.

Molecular analysis

In 96.7% cases it was feasible to start the sequential molecular analysis. The samples obtained were insufficient in the remaining 3 cases: one corresponded to re-staging (difficulty obtaining viable material post neoadjuvant inflammatory changes), and silane coated slides and PEN membrane slides did not contain enough tumour cells for molecular detection in the other two. In the first case, the patient underwent palliative treatment due to deterioration of general condition, while in the other two cases a second procedure was performed [puncture of the primary mass by CT-guided percutaneous transthoracic needle biopsy (CT-guided PTNB) and by EBUS].

IHC was performed in both sufficient and insufficient samples for complete molecular analysis (65.5% vs. 60%, $p = 0.115$).

A partial molecular analysis (EGFR, KRAS, ALK and ROS1) was performed in 78.6% of patients, while complete analysis was carried out in 44% of cases. Table 2 details the individual results for each molecular detection.

Mutations in the EGFR genes were detected in thirteen cases (15%), 12 of them (92.3%) belonging to the adenocarcinoma subtype. Mean age was 67.8 ± 10 years, 38% were women, and 5 cases corresponded to never-smokers. The most frequent mutation was the deletion of exon 19 (54%).

Table 3 Molecular analyses by cytological sample in group 1 (type of mutations: *EGFR*, *KRAS*, *BRAF* and *ERBB2*).

Sample type $n = 87$ (%)	Number of determinations		p
	1–3 ($n = 49$)	4 ($n = 38$)	
Membrane	23 (47.0%)	16 (42.1%)	0.489
Cell block	12 (24.0%)	9 (23.7%)	
Membrane + Cell block	14 (29.0%)	13 (34.2%)	

Molecular analysis by group

In group 1, it was feasible to carry out 4 determinations in 38 (44%), while only 1–3 mutations could be studied in 49 (56%). There were no significant differences in the number of molecular determinations (1–3 vs. 4) ($p = 0.489$) when separated by sampling subgroups between: (1) cell block, (2) PEN membrane slide, and (3) cell block and PEN membrane slide (Table 3).

In group 2, it was feasible (70/81) to carry out 3 determinations (ALK and/or ROS1 and/or MET) in 86%. Of these, complete analysis was performed in 28 cases (34.6%), and only 2 in 38 cases (47%).

Discussion

The results of our study showed that the cytological samples obtained by EBUS-TBNA or EUS-B-FNA, mainly cell blocks and PEN membrane slides, has had a high success rate for sequential molecular analysis, both partially and individually. The College of American Pathologists (CAP), the International Association for the study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) jointly issued guidelines for routine testing of biomarkers in lung cancer and recommended performing the EGFR, ALK and ROS1 genes rearrangements as a first step, and KRAS only if the studies of the first step are negative.[6] In our series, the partial success rate with these 4 biomarkers was around 80%. However, the individual success rate decreased with the sequential analysis, so that EGFR detection was 96.6% while for BRAF it was 67.7%. These results could be explained

by the loss of tumour material during the cell block cuts, scraping tumour cell from PEN membrane slide by manual or laser microdissection, or by the delay in processing fresh slides.[12]

The usefulness of cytological samples obtained by EBUS/EUS-B for molecular analysis has been assessed in different studies, with 2 or 3 molecular alterations studied in most of them.[9,10,13–16] In our series, by applying a protocol for obtaining cytological material, effectiveness was optimal (96.7%) to start sequential molecular analysis, and it was up to 44% favourable for 7 biomarkers. Jurado et al.[13] observed that in 82% (42/56) of cases, enough cytological material was obtained for molecular testing (EGFR, ALK and KRAS), while in our study there were 4 detections (EGFR, KRAS, ALK and ROS1) in 78.6% of cases, which was similar but with an additional biomarker in our series. Folch et al.[16] compared the detection of 3 biomarkers (EGFR, KRAS and ALK) in cytological samples (cell blocks) obtained by EBUS, with samples obtained by other techniques (surgical biopsy of mediastinal and hilar nodes, bronchial biopsies, *CT-guided* PTNB of lung lesions) and managed to show a success rate above 90%, confirming that the EBUS is a suitable tool with which to initiate a genotypic study of NSCLC.

The presence of ROSE was a notable advantage in the optimisation and handling of the samples, as it increases performance in obtaining tumour material for IHC and molecular testing.[17–19] Trisolini et al.[20] demonstrated that the presence of an anatomopathologist can prevent the performance of additional procedures for genotyping in 1 in 10 cases and, although there was no statistical relevance, clinical relevance was observed. In our institution, the good cooperation between cytopathologist and bronchoscopist facilitated the preparation of the samples with adequate tumour material, and in some cases, allowed us to modify the puncture zone in the metastatic lymph node (e.g. presence of necrosis zones), change the lymph node station, or the number of punctures. In our study, ROSE allowed us to perform an average of 3 punctures to obtain sufficient material, below the 4 punctures recommended by the guidelines for obtaining material for molecular study,[21] yet our success rate for partial study was close to 80%.

When evaluating by type of cytological sample (cell block ± PEN membrane slide) for the number of mutations (1–4) we did not observe differences between these samples. PEN membrane slides are an additional tool that contains DNA for molecular subtyping in NSCLC.

The success rate for the detection of EGFR in cytological samples obtained by EBUS varies between 72.2% and 98.7%.[9,10] In our sample, it was 97%, higher than the 90% published by Navani et al.[15] In our study, mutation in EGFR genes was detected in 15%, similar to that reported in histological samples from other Spanish studies (11.6–16.6%).[3,22]

The success rate for the detection of KRAS was 96.3%, higher than the results reported in the studies by Kang and Jurado, 90.2% and 75% respectively[13,23]. Mutations in the KRAS genes were observed in 20%, similar to that reported (23.6%) in different cytological series.[24] In line with previous research, in our experience the presence of EGFR and KRAS mutations was exclusive.[24]

The determination of ALK-rearrangements was possible in 85% of samples, similar to the experience of Jurado et al.,[13] which was 91% (39/41) with a frequency of positive cases (by FISH) of 6.4% (7/109),[14] while in our group only 1 case was positive.

Our article shows that it is feasible to perform a greater number of detections in cytological samples such as cell blocks and membrane PEN slides, following a sequential algorithm and with the help of a cytopathologist. The limitations of our study includes not having all the determinations available in all cases (our assistance protocol did not perform additional analysis in cases where a molecular alteration was already detected) and it was based on data from a single centre with a small sample size, which limits the generalizability of our results.

Immunotherapy with checkpoint inhibitors was recently approved to treat NSCLC. Wang et al.[25] demonstrated 87% feasibility to determine the expression of PD-L1 (programmed death ligand 1) in samples obtained by EBUS-TBNA with a good correlation between the cytological and surgical samples. Likewise, the incorporation of second-generation sequencing (NGS) allowed the analysis of numerous genetic alterations simultaneously, with the use of small amounts of DNA (nanograms). However, its usefulness in cytological samples obtained by EBUS should be validated, since it requires quality DNA and a sufficient tumour cell percentage to give adequate sequencing.[26]

In conclusion, in our series applying a protocol based on obtaining a minimum of 2 cell blocks and optimising the handling of the samples for multiple molecular analysis, we have shown that the cytological samples obtained by EBUS-TBNA or EUS-B-FNA are suitable in a high percentage of patients for individual and partial molecular analysis. PEN membrane slides and cell blocks have shown to be equally valid samples for the determination of mutations.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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REVIEW

Severe asthma in the era of COVID-19: A narrative review

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Abstract
Introduction and objectives: Severe asthma management during the coronavirus disease 2019 (COVID-19) pandemic is a challenge and will continue to be, at least in the next few months, as herd immunity is still a mirage. A lot has to be learned about how COVID-19 affects underlying diseases, and severe asthma is no exception.
Methods: Narrative review of papers available until February 2021 in PubMed and Google Scholar, relating severe asthma and COVID-19. Four main research topics were reviewed: SARS-CoV-2 infection: immunology and respiratory pathology; interrelationship of severe asthma endotypes and COVID-19 disease mechanisms; severe asthma epidemiology and COVID-19; and biologics for severe asthma in the context of COVID-19.
Results: COVID-19 disease mechanisms start with upper respiratory cell infection, and afterwards several immunological facets are activated, contributing to disease severity, namely cell-mediated immunity and antibody production. Although infrequent in the COVID-19 course some patients develop a cytokine storm that causes organ damage and may lead to acute respiratory distress syndrome or multiorgan failure. Regarding severe asthma endotypes, type2-high might have a protective role both in infection risk and disease course. There is conflicting data regarding the epidemiological relationship between COVID-19 among severe asthma patients, with some studies reporting increased risk of infection and disease course, whereas others the other way round. Biologics for severe asthma do not seem to increase the risk of infection and severe COVID-19, although further evidence is needed.

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Conclusions: Globally, in the era of COVID-19, major respiratory societies recommend continuing the biologic treatment, preferably in a self-home administration program.

Introduction

During the coronavirus disease 2019 (COVID-19) pandemic, severe asthma management is a challenge and will continue to be at least in the following months. Despite the recent approval and use of COVID-19 vaccines, the milestone of herd immunity is yet away from reality worldwide.[1] COVID-19 is caused due to the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has caused a substantial increase in hospitalizations for pneumonia with multiorgan disease.[2] COVID-19 first emerged in December 2019[2] and, by the end of 2020, has affected almost every country in the globe, resulting in more than 79 million cases and more than 1.9 million deaths.[3] It is not clear why the clinical presentation may be so distinct, ranging from mild and even asymptomatic to severe clinical conditions such as pneumonia, acute respiratory distress syndrome (ARDS), organ dysfunction and death.[4]

Endemic human coronaviruses present a high homology with SARS-CoV-2.[5] Cross-reactivity exists between these coronaviruses and may explain less severe COVID-19 as reported.[6] Early in infection, SARS-CoV-2 targets cells, such as nasal and bronchial epithelial cells and pneumocytes. Subsequently, the viral inflammatory response is generated, consisting of innate and adaptive immunity (comprising humoral and cell-mediated immunity)[2,7] The pathogenesis of COVID-19 results from an abnormal host response or overreaction of the immune system in some patients with unknown etiology.[7] From a theoretical perspective, asthmatic patients should have increased susceptibility for SARS-CoV-2 infection and to severe COVID-19 due to a deficient antiviral immune response and the tendency for exacerbation elicited by common respiratory viruses.[8] At the beginning of the pandemia, the inclusion of asthmatic patients and patients with other chronic lung diseases in a high-risk population for SARS-CoV-2 infection was based more on common sense than on scientific evidence.[9] Available data at the moment has not shown consistently an expected increased burden of asthmatic individuals among COVID-19 patients.[8]

Severe asthma represents 3—10% of the nearly 400 million asthmatics worldwide but is associated with increased mortality and hospitalization, reduced quality of life and increased healthcare costs.[10] Authorities and physicians are still learning how COVID-19 affects underlying diseases, and severe asthma is not an exception. Even though respiratory viruses are among the most common triggers for asthma exacerbations, not all of these viruses affect patients equally. Available data about whether patients with asthma are at higher risk of being infected with SARS-CoV-2 or having severe forms of the disease is somewhat conflicting.

In the last months, several papers have been published about the relationship between asthma and COVID-19 but a recent review about the particularities and novelties of severe asthma is lacking. Taking into consideration the complexity of severe asthma pathophysiology and the growing knowledge about COVID-19, the authors aim to review four different research topics about the possible interactions between these disease entities:

- SARS-CoV-2 infection: immunology and respiratory pathology.
- Interrelationship of severe asthma endotypes and COVID-19 disease mechanisms.
- Severe asthma epidemiology and COVID-19.
- Biologics for severe asthma in the context of COVID-19.

Methods

The author team generated the topics mentioned above before initiating the review. To answer these questions, a search was performed on PubMed and Google Scholar for papers relating to severe asthma and COVID-19 until February 2020. The search strategy was structured to include terms for "severe asthma" AND "COVID-19″ OR "SARS-CoV-2″. Data was then narratively synthesized by the research topic. Due to the emerging nature of evidence in this field, a broad approach to inclusion was followed, without any study type restriction. All the references judged to have relevant information about prespecified questions were included.

Results

SARS-CoV-2 infection: immunology and respiratory pathology

Upon entry into the host, the inhaled SARS-CoV-2 is likely to bind to epithelial cells in the nasal cavity and start replicating.[11] Angiotensin-converting enzyme-2 (ACE2) is the primary receptor for both SARS-CoV-2 and SARS-CoV.[11] The cellular protease TMPRSS2 also appears vital for SARS-CoV-2 cell entry.[12] There is local propagation of the virus but a limited innate immune response.[11] At this stage, the virus can be detected by nasal swabs and although the viral burden may be low, these individuals are infectious.[11] The virus propagates and migrates down the respiratory tract along the conducting airways, and a more robust innate immune response is triggered.[11] For about 80% of the infected patients, the disease will be mild and mostly restricted to the upper and conducting airway.[13] This phase would be the result of the infection itself. Unfortunately, about 20% of the infected patients will progress and develop pulmonary infiltrates and some of these might develop very severe disease.[13] The virus reaches the lung's gas exchange units and infects alveolar type II cells with high surface expression of ACE2 receptors. The virus rapidly disseminates through

peripheral blood to other organs like the heart, kidney, liver, spleen, etc[14,15] The cytokine storm generated damages the organs or may lead to ARDS or multiorgan failure in COVID-19.[16-18] Human SARS-CoV-2 infection involves the innate immune response, T and B cell immunity and antiviral neutralizing antibodies.

Interrelationship of severe asthma endotypes and COVID-19 disease mechanisms

The current severe asthma approach relies on disease phenotypes identification, but these do not necessarily relate to or give insights into the underlying pathogenetic mechanisms described by disease endotypes.[19] Based on the major immune-inflammatory pathway involved, type2-high, type2-low and mixed endotypes are described for severe asthma.[19]

Type2-high asthma / COVID-19

Type-2 immune responses predominantly mediate the majority of the disease. The type-2 immune response involves T helper (Th) 2 cells, type-2 B cells, group 2 innate lymphoid cells, type-2 macrophages, IL-4-secreting nature killer and natural killer T cells, basophils, eosinophils, and mast cells.[8] A variety of cytokines produced by the immune system and epithelial cells contribute to the regulatory network.[8] For example, IL-4 and IL-13 have essential roles in allergen-specific IgE production and accumulation of Th2 cells and eosinophils in local tissues, as well as epithelial barrier regulation. At the same time, IL-5, IL-9, and IL-13 contribute to eosinophilia and mucus production.[8]

Allergic asthmatic subjects seem to be less likely to be infected by the SARS-CoV-2, which could be due to different factors.[20] The first hypothesis might be related to the anti-inflammatory effect of the inhaled corticosteroids (ICS) and their negative impact on the cytokine storm elicited by the virus.[20] This effect might be boosted by increased compliance with asthma treatment driven by COVID-19 fear.[20] Individuals with allergic asthma have confirmed reduced ACE2 expression[21] and in vitro experiments showed that IL-13 also reduced the ACE2 expression.[21] Interestingly, ICS were associated with lower expression of ACE2 and TMPRSS2 after adjustment for asthma severity and may explain the low prevalence of asthma among COVID-19 patients in some studies.[22] This fact may justify why data about the relationship between asthma and susceptibility to SARS-CoV-2 is contradictory, as compliance with ICS might be an unconsidered confounding factor.[22] Furthermore, some ICS have less peripheral airway deposition and might affect less ACE2 expression of type 2 pneumocytes.[22] Another critical point is the smoking status of the patients.[22] ACE2 expression in bronchial biopsies was found to be higher in smokers, suggesting an enhancing effect on SARS-CoV-2 entry into lung cells.[22]

Certain aspects of the type-2 immune response, including type 2 cytokines (IL- 4, IL-13, etc.) and accumulation of eosinophils, might provide potential protective effects against COVID-19 due to its anti-inflammatory effects.[8,23] For example, IL-4 can suppress type-1 immune response.[8] This effect is obtained not only by suppression of Th1 cells development but also by the inhibition in the production of multiple pro-inflammatory cytokines associated with type-1 immune response, including IL-1β, TNF-α, IL-6, and IL-12.[8] It has also been shown that IL-13 has immunoregulatory effects through inhibiting the secretion of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and chemokines (IL-8, MIP-1α and MIP-1β, and monocyte chemotactic protein-3).[8] Additionally, IL-13 mediated type-2 inflammation has a significant role in ACE2 downregulation and TMPRSS2 upregulation.[7,22] Furthermore, IL-9 reduces TNF-α and IL-10, but increases the secretion of TGF-β on LPS-activated monocytes.[8] It is possible, that predominance of type 2 cytokines might counteract the accumulation of pro-inflammatory cytokines, including the pathogenesis of COVID-19.[8]

Eosinophils cause tissue damage by releasing many toxic proteins and other preformed pro-inflammatory mediators after degranulation processes.[24] Eosinophils can actively promote type 2 immune responses by producing a range of immunoregulating cytokines and other factors.[24] Although eosinophils may have protective effects on different viral infections, their role in SARS-CoV-2 is incompletely understood and may even be absent as the antiviral activity has not yet been demonstrated in humans.[21,25] Many COVID-19 patients present eosinopenia, although it is not reported in all cohorts[21,24] This phenomenon is probably secondary and not directly contributing to disease course.[21,24] The immune mechanism of eosinopenia in COVID-19 remains unclear. Still, it is probably multifactorial, involving inhibition of the eosinophil life cycle's main steps, apoptosis induced by type 1 IFN during the acute infection or relation to eosinophil consumption by eosinophil antiviral actions.[24] Increased tissue migration is unlikely because eosinophils infiltration was not found in pulmonary tissue of COVID-19 patients, but further research is needed.[21] An important fact is that eosinophil levels improved in patients before discharge, suggesting that eosinophil resolution may be an indicator of improvement of clinical condition.[24]

Type-2 low asthma / COVID-19

Type2-low severe asthma is a distinct endotype with relevant features such as increased severity and remodeling and less satisfactory response to anti-inflammatory treatment.[19] The understanding of these disease mechanisms lags far behind type2-high endotype.[19] Several pathways were evaluated, such as the dysregulated innate immune response, including intrinsic neutrophil abnormalities, the inflammasome pathway and the activation of the IL-17 pathway.[19] In severe asthma with predominantly neutrophilic phenotype, transcobalamin-1, metalloproteinase (MMP) 9, mucins, and oxidative stress responses are upregulated.[19] Many severe asthma facets are mechanistically associated with Th17 cell-derived cytokines and other immune factors that mediate neutrophilic influx to the airways and remodeling.[19] TGF-β1 is a pivotal mediator involved in airway remodeling that correlates with enhanced Th17 activity and is essential for Th17 differentiation and IL-17A production. IL-17A can reciprocally enhance activation of TGF-β1 signaling pathways, whereas combined Th1/Th17 or Th2/Th17 immune responses additively impact severity.[19] Type2-low and IFN-high individuals were found to express a high level of ACE2, and this might increase the risk of SARS-CoV-2 infection[22] but further evidence is needed to corroborate this finding.

Neutrophils are one of the predominant lungs infiltrating leukocytes in severe COVID-19 and neutrophilia predicts poor clinical outcomes.[21] Post-mortem analysis of lung samples from

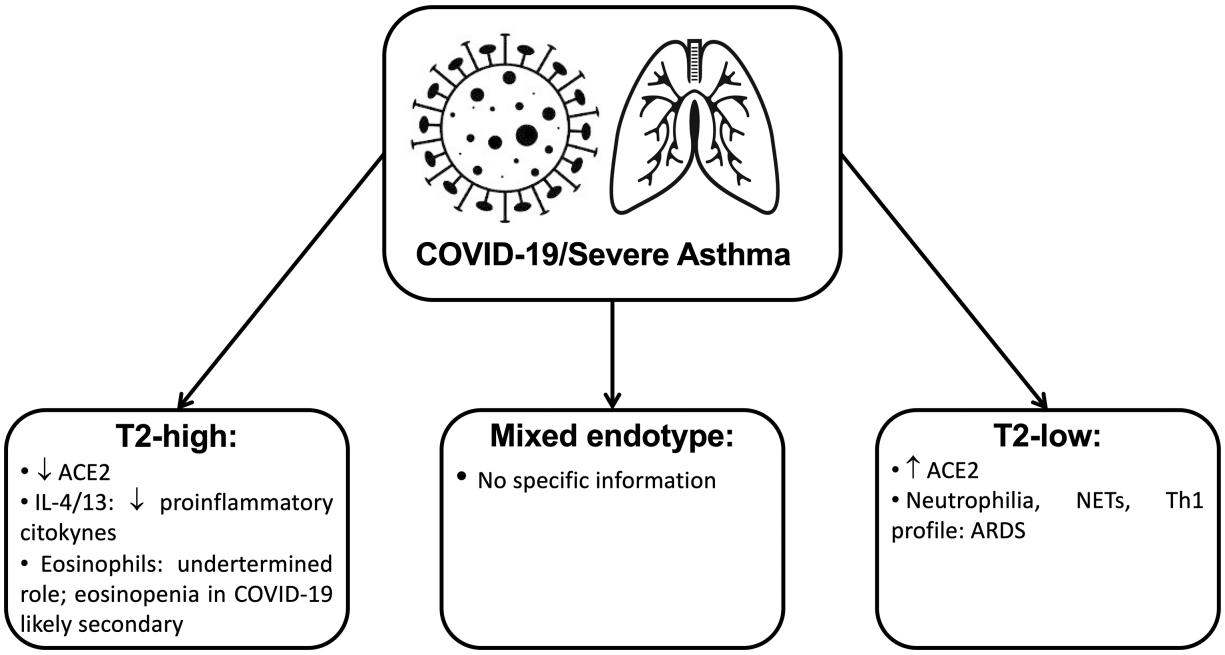

Figure 1 Summary of the main evidence about severe asthma endotypes and COVID-19 disease mechanisms. Legend: ACE2: angiotensin-converting enzyme 2 receptor; COVID-19: coronavirus disease 2019; IL: interleukin; NETs: neutrophil extracellular traps; Th1: T Helper 1 cell.

COVID-19 patients showed neutrophil infiltration in pulmonary capillaries and neutrophil extravasation into the alveolar space, and the inflammatory profile observed in lungs from COVID-19 patients is Th1, Th17 phenotype.[21,24] In experimental models of smoke-induced acute respiratory distress, a Th17/neutrophilic syndrome, ACE2 was upregulated.[26] A type 2-low endotype can be an aggravating factor to COVID-19 severity.[27]

Under neutrophil-activating conditions, such as those occurring during systemic inflammation, neutrophil extracellular traps (NETs) can be released.[28] Although this is a way to trap pathogens, NET formation is linked to pulmonary diseases, particularly ARDS.[28] Severe COVID-19 conditions with uncontrollable progressive inflammation presumably induce intense crosstalk between neutrophils releasing NETs and IL-1β secretion from macrophages.[28]

Mixed endotypes asthma / COVID-19

At the present moment, we could not find any information relating to mixed endotypes of severe asthma and COVID-19 and this is an area of particular research interest.

Fig. 1 is a summary of the main evidence about severe asthma endotypes and COVID-19 mechanisms:

Severe asthma epidemiology and COVID-19

The literature search yielded 56 records and after identifying and removing duplicates 35 articles remained. After screening for relevant titles/abstracts, 11 publications were considered to report original epidemiological data. Four of these publications were related to Severe Asthma Registries.

In the Belgian Severe Asthma Registry[29] only 14 out of 676 patients (2.1%) had SARS-CoV-2 infection confirmed by a positive PCR or serology testing. These findings indicated a lower incidence than in the general population (5.1%). There was no statistically difference in COVID-19 incidence between patients treated or untreated with biologics. No deaths were attributed to COVID-19 on this cohort of severe asthma patients.

In the Severe Asthma Network in Italy (SANI)[30], Heffler et al. reported an incidence of 26 confirmed or highly suspected infections out of 1504 patients (1.73%). The majority ($n = 21$) were under biologics: 15 on anti-IL-5 and six on anti-IgE. As previously described, due to the higher proportion of COVID-19 infections with anti-IL-5 treatments, the authors speculated about the role of these monoclonal antibodies (mAbs) in the COVID-19 course. It should be noted that this finding was not seen in the Belgian registry.[29] In the Italian cohort, the related mortality was 7.7%, lower than in the general Italian population (14.5%).

In the Italian Registry of Severe Asthma (IRSA)[31], a different Italian severe asthma registry, seven subjects out of 558 (1.25%) contracted COVID-19. The hospitalization rate in COVID−19 infected patients with severe asthma was similar to the general population, and no deaths were reported. The proportion of COVID-19 in patients on biologics was 5.43% compared to 0% on subjects treated with ICS−LABA alone, but no statistical difference was found.

Eger et al.[32] found a frequency of nine out of 634 (1.4%) patients with COVID-19 among severe asthma patients on biologics in the Dutch Severe Asthma Registry RAPSODI. From these, five patients needed intensive care and one patient died. In the group of 73 patients not prescribed any biological, one (1.73%) was diagnosed with COVID-19. In this study, the proportion of COVID-19 related hospitalization, intubation, and death was 1.26% in the patients under biologics, which is a number 4.5 times higher than in the Dutch population within the same age category (0.28%). It must be

considered that RAPSODI patients under biologics had a higher prevalence of obesity than the general population (30% versus 15%).

Rial J. et al.[33] conducted a multicentre study in nine centres from the Spanish Severe Asthma Network. Among 545 adult severe asthma patients under biological treatment, 35 (6.4%) had COVID-19, a proportion similar to the Spanish seroprevalence (5.2%) at the time of the study. Eight patients were hospitalized and one patient died. No statistical differences were found between the frequency of COVID-19 in patients under anti-IgE (5.32%) compared to the subjects treated with anti-IL-5 (7.4%).

Table 1 presents a summary of the epidemiological data regarding COVID-19 in the European severe asthma registries and the Spanish Severe Asthma Network.

Caminati et al.[34] reviewed the medical records of patients admitted to COVID-19 Units of six Italian cities ($n = 2000$). From the 42 asthmatic patients identified, patients on GINA 4/5 and those not adequately treated were considered at higher risk. Hauron-Diaz et al.[35], in a sample of 80 severe asthma patients followed in the Allergy Service of Infanta Leonor University Hospital (Madrid, Spain), SARS-CoV-2 infection was confirmed in three (3.75%) patients. None of the patients required intensive care. Chibba et al.[36] conducted a retrospective study that assessed the risk of hospitalization associated with asthma and/or inhaled corticosteroid use in patients with COVID-19. The proportion of asthmatic patients using ICS plus long-acting beta2-agonists admitted to an intensive care unit was higher (57.9%) than those using ICS alone. Kow et al.[37] suggest that this finding may indicate that those with more severe disease have worse outcomes. According to the health analytics platform OpenSafely[38] that covers 40% of all patients in England, severe asthma (defined as asthma with recent use of an oral corticosteroid) was associated with COVID-19 related death after adjusting for sex and age (hazard ratio: 1.13; 95%: 1.01−1.26).

Calmes D. et al.[39] conducted a study to evaluate if obstructive diseases were risk factors for intensive care unit stay and death due to COVID-19. Twenty out of 57 (35%) asthmatic patients included were under a high dose of inhaled steroids, and 2 (3%) were taking oral steroids daily. In this study, inhaled and oral corticosteroid treatment were not identified as risk factors.

Biologics for severe asthma in the context of COVID-19

There are now five approved biologic agents for severe asthma: one anti-IgE — omalizumab, two anti-IL-5 - mepolizumab and reslizumab, one anti-IL-5 receptor alpha (Rα) — benralizumab - and the anti-IL-4Rα dupilumab.[40]

Omalizumab is a humanized monoclonal antibody that selectively binds to human IgE, preventing its high-affinity receptor.[41] Regarding anti-IL-5/IL-5-R, their major effect is the reduction or depletion of tissue and peripheral blood eosinophils.[41] Mepolizumab and reslizumab are humanized anti−IL-5 mAbs that bind circulating IL-5 with high affinity and prevent binding of IL-5 to its receptor.[41] Benralizumab is a humanized afucosylated mAb that binds to the alpha subunit of the human IL-5Rα, specifically expressed on the surface of eosinophils and basophils.[41] Dupilumab is a mAb that

Table 1 — Epidemiological data regarding COVID-19 in the European severe asthma registries.

Author	Study population	Deadline of the study	Number of participants	Proportion of COVID-19+ patients		Proportion of deaths on COVID-19+ patients	
				Severe asthma	General population	Severe asthma	General population
Hanon et al. ([29])	Belgian Severe Asthma Registry	8th July 2020	$n = 676$	2.1%	5.1%	0%	15.3%[1]
Heffler et al. ([30])	Severe Asthma Network in Italy (SANI)	19th June 2020	$n = 1504$	1.73%	0.39%[1]	7.7%	14.5%
Antonicelli et al. ([31])	Italian Registry of Severe Asthma (IRSA) — until 18th May 2020	18th May 2020	$n = 558$	1.25%	0.39%[1]	0%	14.2%
Eger et al. ([32])	Dutch Severe Asthma Registry (RAPSODI)	30th April 2020	$n = 634$	1.4%	0.23[1]	1.25%	0.28%
Rial J et al. ([33])	Spanish Asthma Network (nine centers)	June 2020	$n = 545$	6.4%	5.2%	0.18%	11.1[3]

1- Data not reported by the authors. Calculated using the data provided by World Health Organization (https://covid19.who.int/region/euro/country) at the time of the deadline of the study period.

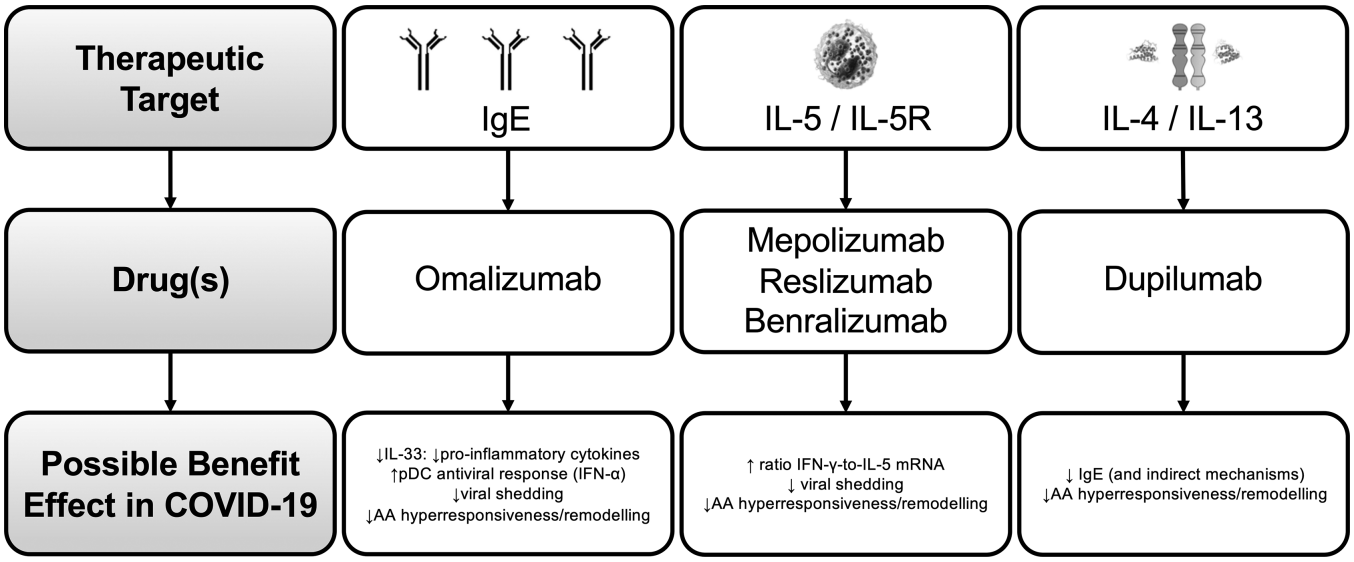

Figure 2 Summary of the potential main benefits of monoclonal antibodies for severe asthma treatment in COVID-19 Legend: AA: airways Ig: immunoglobulin; IFN: interferon; IL: interleukin; pDC: plasmacytoid dendritic cells.

attaches to the alpha subunit of IL-4/13 receptors and promotes signaling after binding to the IL-4Rα subunit.[41]

MAbs targeting type-2 inflammation is likely to reduce the risk of COVID-19 mediated severe asthma exacerbations by reducing baseline airway inflammation and possibly through specific antiviral properties.[20] Omalizumab, cross-linking IgE, would lead to lower type 1 IFN production.[20] Mepolizumab, reslizumab and benralizumab, act by increasing the ratio of IFN-γ-to-IL-5 mRNA, which is associated with lower viral shedding and faster disease clearance.[20] Finally, IL-4 is crucial for antibody switching to IgE, and IL-13 is a Th2 cytokine involved in airway hyperresponsiveness and remodeling; both of them are involved in susceptibility and clearance of viral infections affecting lower airways.[20]

Fig. 2 shows a summary of the potential main benefits of mAbs for severe asthma treatment in COVID-19:

From another perspective, it is theoretically reasonable that mAbs targeting type-2 asthma inflammation can be associated with increased risk for COVID-19 (in terms of infection or severity). However, early reports did not show this clearly.[42] Available data did not show consistently significant differences among patients treated with different mAbs in a large cohort study.[42] Even though, contradictorily, in the Severe Asthma Network in Italy (SANI), patients treated with anti-IL-5 mAbs had a considerably higher proportion of SARS-CoV-2 (71%) compared to those treated with anti-IgE (29%)[30] and this possible effect about increased disease severity in patients treated with biologics for type-2 inflammation has been further debated in another recent study[32]. Although the number of cases was too small to draw any definitive conclusions, the authors speculated that different mAbs could have specific and distinct impact on antiviral immune response, as suggested for anti-IgE as protective for other viral infections.[30] Furthermore, the authors also proposed that the consequence of eosinopenia induced by anti-IL-5 agents might be a risk factor for more severe COVID-19. Currently, no large series of severe asthmatics treated with biologicals infected by COVID-19 have been published, so the ideas about the role of mAbs in modulating the risk of COVID-19 are speculations and need further evidence.[30] Assuming the previously stated notion that eosinopenia associated with COVID-19 is likely to be a secondary phenomenon, this concern about biological drug-induced eosinopenia may not be relevant.[25] Comprehensive and large-scale investigations are expected to elucidate further the interactions between COVID-19 and type-2 high severe asthma.

Treatment with omalizumab might protect from severe forms of COVID-19[43]. Omalizumab was shown to enhance antiviral immunity via downregulation of the high-affinity IgE receptor on plasmacytoid dendritic cells, essential for antiviral immune responses.[43] Cases of omalizumab patients who contracted COVID-19 have been recorded, and no increased susceptibility to severe disease or asthma exacerbations was observed[43,].[44] The PROSE study showed that in severe asthmatics, omalizumab treatment decreases the duration of viral infections, viral shedding and the risk of respiratory viral illnesses.[45] Another study indicated that omalizumab treatment augments plasmacytoid dendritic cells IFN-α responses and attenuates the FcεRIα protein expression induced by these cells, reducing the susceptibility to virus-induced asthma exacerbations.[46] Besides, IL-33 levels decrease after omalizumab treatment[47,48] and this interleukin is important for the production of pro-inflammatory cytokines (including IL-6, IL-1β, TNF-α, MCP-1, and prostaglandin D2).[49] All this data suggests a potential effect of omalizumab on antiviral responses.[8] It would be interesting to explore whether previous or concurrent use of omalizumab might have protective effects on COVID-19 infection, either on duration, severity or both.[8]

In those on mepolizumab therapy, a very recent publication reports the outcomes of four severe asthmatic patients with COVID-19 while receiving treatment with mepolizumab, from different centres (UK, Italy and North America).[50] Only one patient (who had recognized risk factors for admission and death from COVID-19) required hospitalization and

ventilatory support but recovered without evidence of long-term respiratory consequences.[50] Four published case reports describe six patients contracting SARS-CoV-2 while receiving benralizumab treatment for severe eosinophilic asthma. The range of symptoms experienced by each patient varied, but all of them recovered.[51-53] These reports add to the debate about whether patients with eosinophil targeting therapies might have an unaltered outcome in COVID-19. As previously mentioned, evidence of severe asthma registries still poses this into question.[32] Considering the reported evidence, it is clear that more data is needed. Until then, and for the moment, in severe eosinophilic asthma the expert recommendations are to continue biologic therapy unchanged.[54]

Case reports of severe asthma patients under dupilumab show no association with negative impact in COVID-19.[55,56] However, as previously mentioned, dupilumab will block IL-4, which is fundamental for the differentiation of Th2 by IL-6 and this might shift the balance Th1/Th2 towards Th1 and facilitate INFγ production.[57] The differentiation of Th2 by IL-6 is dependent on endogenous production of IL-4 whose activity is significantly reduced by dupilumab.[57] This mechanism plays a central role in the "cytokine storm" damaging lung in COVID-19 patients[57] but the clinical consequences are still a matter of debate[32,56] On the other hand, due to the mechanism of action of dupilumab, drug discontinuation could be associated with higher susceptibility toward infections, even if there is no evidence supporting this hypothesis regarding SARS-CoV-2.[57] Considering available evidence at the moment, treatment with dupilumab should not be stopped during the COVID-19 pandemic.

Globally, in the era of COVID-19, major respiratory societies (Global Initiative for Asthma, European Respiratory Society, British Thoracic Society and American Academy of Allergy, Asthma & Immunology) recommend continuing the biologic treatment, preferably in a self-administered home program.

Discussion

Evidence about COVID-19 is rapidly evolving, and data connecting asthma and COVID-19 is also trendy. A recent systematic review and meta-analysis about asthma and COVID-19 suggested that the prevalence of asthma among COVID-19 patients is similar to the global prevalence of asthma.[58] Another interesting conclusion from this study is that people with asthma have a lower risk than those without asthma for acquiring COVID-19 and have similar clinical outcomes.[58] Despite the increasing number of published studies about COVID-19 and asthma, the knowledge gap for severe asthma persists due to its particularities. The relationship between COVID-19 and severe asthma is nowadays still a matter for debate as conflicting evidence is published. In the following months with the burden of COVID-19 increasing in terms of mortality and healthcare resources utilization, severe asthma patients will face a hard challenge.[3]

The COVID-19 pandemic disrupted science in 2020 with a sharp increase in articles on all subjects being submitted to scientific journals and severe asthma was not an exception.[59] This vortex of global research has mixed consequences.[60] Positives include the higher provision of open access to COVID-19 studies, increased collaboration, expedited governance and ethics approvals of new clinical studies, and broader use of preprints.[60] But many challenges have become evident.[60] Before the pandemic, it was estimated that up to 85% of research was wasted because of poor questions, poor study design, regulation inefficiency, and no or insufficient reporting of results.[60] Many of these problems are amplified in COVID-19 research, with time pressures and inadequate research infrastructure contributing. This might also contribute to the previously discrepancies about COVID-19 impact on asthma and severe asthma in particular.[60,61] At the present moment, no definitive conclusions can be drawn as many confounding factors might have influenced available evidence.[61] Interestingly, as time goes by, several aspects apart from the disease itself start to be shown about the impact of COVID-19 pandemic on severe asthma patients. A recent study concluded that during this period, severe asthma patients were significantly more impacted by the pandemic with increased rates of unemployment and difficulty in getting asthma meds compared to those with non-severe asthma.[62]

Recent publications have discussed the concept of a long-COVID syndrome that is common and independent from the severity of the acute COVID-19 syndrome.[63] This syndrome is more frequent in women and may not be directly attributable to the effect of SARS-CoV-2 but rather an interaction of biopsychosocial effects.[63] A recently published meta-analysis studied the frequency of potential respiratory symptoms in COVID-19 patients.[64] Fatigue (52%), dyspnea (37%), chest pain (16%) and cough (14%) were the most frequently reported persistent symptoms among COVID-19 pneumonia survivors.[64] Another recently published meta-analysis described that COVID-19 patients with pneumonia have long consequences in lung function and the most important one is the diffusion capacity affection.[65] How these pathophysiological and symptomatic changes in long-COVID interact with severe asthma is still uncertain.

Anticipating that there are at least several months of pandemic still ahead and that SARS-CoV-2 will be circulating for a longer time, severe asthma patients need proper answers about their disease management in this context. Large-scale, preferably multinational real-life studies with detailed information on asthma phenotype and medication usage in patients with a confirmed diagnosis of COVID-19 would be an ideal next step to further build on this new evidence.[61] One critical point is that the most relevant evidence about severe asthma and COVID-19 comes from studies published in mid-2020 and as the number of patients increases the data becomes more robust. From our point of view, apart from the previously discussed aspects, during this COVID-19 pandemic several questions for both patients and physicians involved in severe asthma management are at the forefront of everyone's mind, namely:

- What are the long-term effects of past COVID-19 infection on asthma disease progression?
- What is the real dual role of steroids used for severe asthma management in COVID-19 (cytokine blocking versus induction of coronavirus replication)?
- What are the possible interactions between mAbs directed for COVID-19 treatment and mAbs for severe asthma treatment?

Conclusions

Severe asthma management during the COVID-19 pandemic is now a challenge that will continue in the near future until herd immunity is reached. COVID-19 disease mechanisms affect severe asthma patients and definitely, the disease endotypes might confer different responses. The immunological consequences of SARS-CoV-2 infection are broad and complex, contributing to the increasing complexity of severe asthma knowledge. Regarding severe asthma endotypes, type2-high seems to have a protective role both in SARS-CoV-2 infection and COVID-19 course. On the other hand, type-2 low seems to confer an increased risk of infection and severe disease forms. From a theoretical point of view, although this idea might be defensible further robust evidence is needed to corroborate it. Biologics for severe asthma do not seem to increase the risk of infection and severe COVID-19 and might even be protective, although further evidence is needed. Globally, in the era of COVID-19, major respiratory societies recommend continuing the biologic treatment, preferably in a self-administrated home program.

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Declaration of Competing Interest

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REVIEW

A snapshot of exhaled nitric oxide and asthma characteristics: experience from high to low income countries

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Abstract Nitric oxide is a gas produced in the airways of asthmatic subjects and related to T2 inflammation. It can be measured as fractional nitric oxide (FeNO) in the exhaled air and used as a non-invasive, easy to evaluate, rapid marker. It is now widely used in many settings to determine airway inflammation. The aim of this narrative review is to report relationship between FeNO and the physiopathologic characteristics of asthmatic patients. Factors affecting FeNO levels have also been analysed as well as the impact of corticosteroid, target therapies and rehabilitation programs. Considering the availability of the test, spreading this methodology to low income countries has also been considered as a possibility for evaluating airway inflammation and monitoring adherence to inhaled corticosteroid therapy. PubMed data search has been performed restricted to English language papers. Research was limited to studies in adults unless studies in children were the only ones reported for a particular issue.

This revision could be useful to summarize the role of FeNO in relation to asthma characteristics and help in the use of FeNO in different clinical settings particularly in low income countries.

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Introduction

Asthma is a heterogeneous disease usually characterized by airway inflammation.[1] Inflammation characterizes most of the steps of the disease, and is strictly associated with airway remodelling.[2] Skewing towards type 2 inflammation is usually a characteristic of asthma with eosinophils represented as the most common cells recruited in the airways.[3] This type of inflammation is treatable with inhaled corticosteroids (ICS), associated with long acting beta 2 agonist (LABA).[4] However, severe asthmatic patients at the top of ICS + LABA prescriptions experience frequent exacerbations, which need oral corticosteroids.[1] Introduction of monoclonal antibodies targeting IgE, IL-5, IL-5R and IL-4Rα successfully reduce T2 inflammation in these patients, avoiding in most of the cases, oral corticosteroid use.[5]

The evaluation of inflammation in asthmatic patients may be useful at time of diagnosis, in order to define the inflammatory pattern of the patient, and even more useful during the follow-up to assess patient's adhesion to therapy, response to treatment and necessity of step-up or down of therapy. Furthermore, in patients with severe asthma not controlled by high dose ICS, the evaluation of the type of airway inflammation is useful to select candidates for monoclonal therapy.[1]

Many efforts have been focused in recent years on trying to detect T2 inflammation with distinct biomarkers in different specimens. Biopsies and bronchoalveolar lavage were the first used to assess airway inflammation but, due to the invasiveness of the procedures, their use is limited to research or to special cases. Induced sputum and fractional exhaled nitric oxide (FeNO) measurements are the most reliable non-invasive procedures to assess T2 airway inflammation.[6,7] Induced sputum allows us to monitor airway inflammation in asthmatic patients and to reduce exacerbations when used to modulate ICS therapy, evidence A.[1] Although reproducible and standardized by International guidelines,[8,9] induced sputum diffusion is relatively time consuming and limited to specialized centres. Efforts have been made to simplify this technique and spread it in less developed countries.[10] Nitric oxide (NO) is a gas produced in the airways and detectable as exhaled NO. Its production increases in the airways when T2 inflammation is present; therefore, measurement of FeNO is a non-invasive technique to highlight T2 airway inflammation. FeNO evaluation is certainly more widespread in different clinical settings than induced sputum because it does not require particular equipment, apart from the specific analyser. Furthermore, induced sputum technique is time-consuming, needs staff training not only for the collection but also for the processing of samples.

In recent decades, different studies evaluated the correlation between FeNO and sputum eosinophils in order to use this biomarker as a surrogate for sputum eosinophils even in low income countries. The correlation between these two biomarkers is significant but weak since sputum eosinophils and FeNO only partially share T2 mechanisms of inflammation.[11] Blood eosinophils have also been used in recent years as surrogate marker of sputum eosinophils[12] with different accuracy with respect to FeNO when sputum eosinophils were used as ''gold standard'' for eosinophilic airway inflammation. Fig. 1 summarizes the most consolidated and widespread methodologies to assess airway inflammation. Exhaled breath condensate has been widely used for research in airway inflammation with the evaluation of different mediators such as H_2O_2, 8-isoprostane, adenosine, pH and leukotrienes, but as reported by the European Respiratory Society (ERS) task force for exhaled biomarker in lung diseases, standardization of collection, storage and evaluation procedure are needed for this methodology.[13]

FeNO has recently been the topic of review papers, but to the best of our knowledge, this is the first review trying to summarize the relationship between FeNO, asthma characteristics, interfering factors and therapeutic approaches, rehabilitation programs included. It also considers the scaling up of this methodology to low income countries.

Methods

We conducted a literature search in PubMed to find studies focused on asthma characteristics and FeNO in order to evaluate which the relationships reported between this biomarker and characteristics of the disease. The search terms were: FeNO (or fractional exhaled nitric oxide) AND asthma in all fields of search AND hyperreactivity OR hyperresponsivness, OR airway remodelling, OR infection, OR air pollution, OR smoking, OR exercise, OR comorbidities (atopic dermatitis, nasal polyposis, etc.), OR occupational asthma, OR developing countries, OR rehabilitation programs. We considered English papers from 1994 to February 2020 and studies in adult subjects, unless those in children were the only reported on a particular issue.

Results

FeNO definition

Nitric oxide is a gas present in the airways and detectable as fractional nitric oxide (FeNO) in the exhaled air. High FeNO levels are found in a sub-group of asthmatic patients with T2 inflammation. Inducible nitric oxide synthases (iNOS) are the enzymes responsible for the increase of NO in the airways due to inflammation. Analysis of bronchial epithelial different gene expression in asthmatic patients revealed different clusters of subjects with high or low FeNO levels suggesting different molecular pathways.[14] Measurement of FeNO at different flows allows us to evaluate the amount of this gas produced by central and peripheral airways and to obtain selective information regarding inflammation at different sites of the airways. Differences between electrochemical and chemiluminescence based analysers in FeNO levels have recently been reported,[13] proposing an equation to convert one level to another.[15]

FeNO use in asthma

Increased FeNO levels in asthmatic patients were reported many years ago.[16] American Thoracic Society (ATS) /ERS guidelines summarized data on FeNO measurement showing that FeNO levels >50 ppb are representative for T2 inflammation in adults, while FeNO<25 ppb for lack or suppression

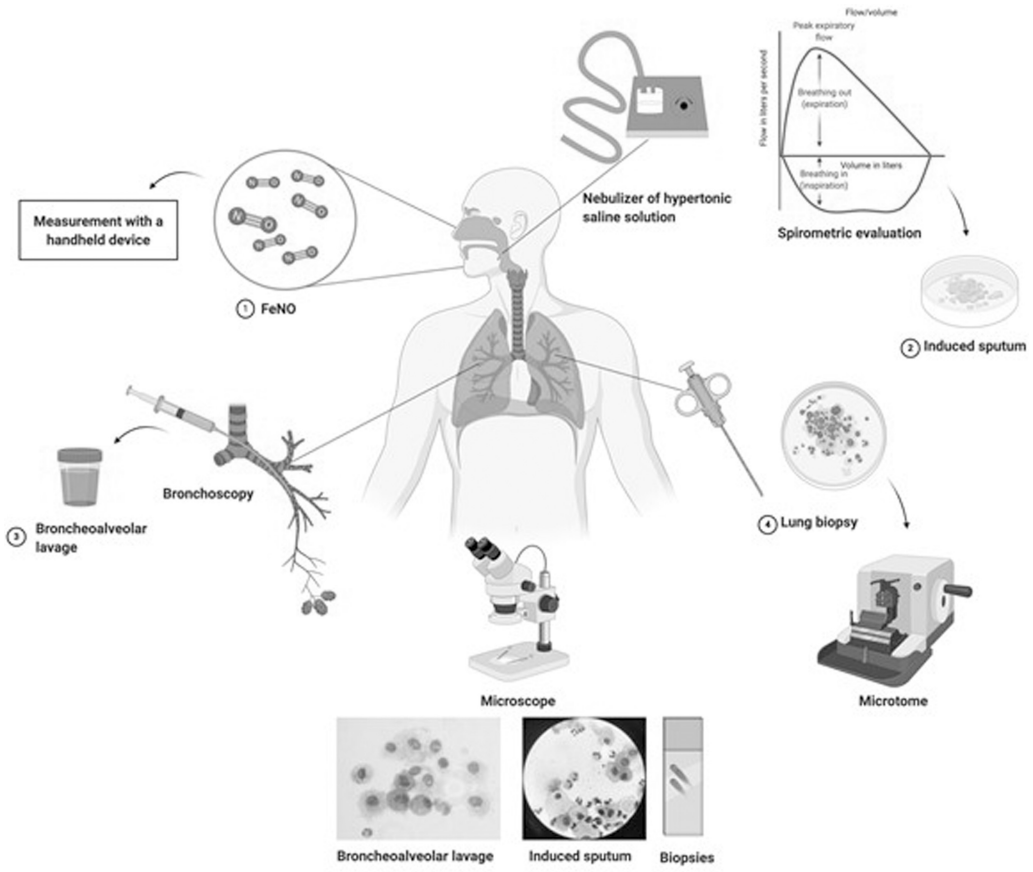

Figure 1 Most used methodologies to assess airway inflammation, from the least to the most invasive ones. 1. FeNO can be measured with a handled device, 2. Induced sputum needs an ultrasonic nebulizer, serial spirometric evaluation during the inhalation period to avoid excessive bronchoconstriction and sample processing in the laboratory, 3. Bronchoalveolar lavage is performed during a broncoscopy procedure and needs a processing of the sample in the laboratory, 4. Biopsy, with close or open methods, is the most invasive procedure used only in selected cases, less frequently to evaluate airway inflammation. Figure was created with Biorender.com.

of it, intermediate levels should be cautiously evaluated considering possible factors lowering (smoking habit) or increasing (atopy) FeNO levels.[17] The positive predictive value (PPV) for FeNO>25 ppb to predict sputum eosinophils >3% is 45% while for FeNO > 50 ppb PPV increases to 77%. Negative predictive value of FeNO is probably more useful (88% and 83% respectively),[18] particularly considering that application of these cut-offs in real life run into many subjects with intermediate levels (25–50 ppb).[18] FeNO levels relate more closely to the risk of disease exacerbations than to disease severity. The ability of FeNO to predict airway inflammation varies as opposed to airway calibre, therefore, FEV_1 reduction with involvement of peripheral airways can be associated with low FeNO levels even if airway eosinophilic inflammation is high,[19] on the contrary, bronchial obstruction limited to central airways can be associated with increased FeNO levels. FeNO levels may identify non-smoker patients with not well-controlled asthma while sensitivity of FeNO seems lower than evaluation through ACT and ACQ.[20]

In mild allergic asthma FeNO levels decrease after a specific inhalation challenge together with bronchoconstriction, they return to normal levels after 8 h and again increase 24 h after the challenge.[21]

FeNO levels correlate with asthma exacerbations in severe asthmatic patients independently of blood eosinophils and serum periostin levels.[22,23] Since cough is a frequent asthma symptom, which can be associated with high T2 inflammation, cough variant asthma can be suspected when cough and high FeNO levels are present.[24]

Atopy is associated with increased FeNO in children while in adult subjects FeNO levels increase when allergic rhinitis is present.[25] Age and height are also positively correlated with FeNO levels.[26] FeNO levels are associated with a rapid lung decline in patients with difficult to treat asthma.[27]

FeNO and characteristics of asthma

Bronchial hyperresponsivness

Bronchial hyperresponsivness (BHR) is a characteristic of asthmatic patients not always associated with T2 inflammation.[28] Increased FeNO levels, independently of increased blood eosinophils, correlate with BHR in young asthmatic patients and the simultaneous increase of FeNO and blood eosinophils increases the risk of BHR.[29] Very high FeNO levels (> 100 ppb) are strong predictors of bronchial hyperreactivity in Asian patients with suspected asthma.[30]

Furthermore, FeNO has been proposed as predictive marker of bronchial hyperreactivity to both mannitol and bradykinin in asthmatic patients.[31,32]

Bronchial reversibility

Different stimuli, both specific and non-specific can trigger bronchoconstriction in asthmatic patients.[1] FeNO levels might be lower than real when bronchoconstriction is present.[33] As recommended by ATS/ERS guidelines, FeNO should be measured before spirometric manoeuvres.[17] Albuterol inhalation in steroid-naïve patients, determined increased FeNO levels.[34] In a cohort of non-smoking asthmatics, baseline FeNO levels correlated with bronchial reversibility,[35] and with change in FEV_1 occurred after a reversibility test in patients treated with ICS/LABA.[36] In subjects with asthma symptoms but with absence of reversibility, FeNO >32 ppb can predict positivity to methacholine test with high specificity (85 %) and lower sensitivity (47%).[37]

Airway remodelling

Remodelling is a complex and multifactorial event characterized by increased thickness of the reticular epithelial membrane, hypertrophy of airway smooth muscle cells, hyperplasia of goblet cells and angiogenesis processes.[38] Since remodelling is often present together with inflammation, it is rather difficult to evaluate the contribution of NO directly to the remodelling process. High FeNO levels could be related to increased bronchial wall NO concentration due to airway inflammation or altered bronchial diffusivity of NO due to remodelling.[39]

Many years ago, Mahut et al. found in children with refractory asthma that alveolar nitric oxide correlated with TGF-β, a well-known pro-fibrotic cytokine, reticular membrane thickness, tissue inhibitor metalloproteinase (TIMP)/metalloproteinase 9 in BAL and with MEF_{25-75}.[40] Structural changes in the airways of asthmatic patients seem to be correlated with FeNO levels. Alveolar NO represents the production from the seventeenth to the twenty-third generation of bronchi, while central airway NO is produced from the first to the sixteenth generation.[41] In adult asthmatic patients FeNO levels correlate with wall thickening in the central area, suggesting a role particularly with the remodelling of this tract.[41] Therefore, differentiation between NO derived from central or peripheral airways could be useful when airway remodelling is studied. Treatment with corticosteroids after exacerbations slightly decreased FeNO levels without affecting bronchial wall thickening in moderate/severe persistent asthmatics,[42] confirming an active role of ICS on the amount of NO produced by bronchial walls and associated to inflammation.

Characteristics of airways in asthmatic patients and FeNO levels are summarized in Fig. 2

Comorbidities

Asthmatic patients often have one or more comorbidities and FeNO levels could change depending on how comorbidities affect airway inflammation (Table 1). The prevalence of chronic rhinosinusitis with nasal polyposis is high in severe asthmatic patients (40.6%).[43] FeNO is a good predictor of nasal polyposis in severe asthmatic patients even when blood eosinophils are normal or low.[44]

Obesity is a comorbidity with impact on lung function, particularly in asthmatic patients and any effort towards weight loss could improve disease severity and quality of life.[45] Adipokines produced by adipose tissue determine a low grade of inflammation, which also affects the lungs.[46] This type of inflammation, mainly characterized by increased IL-6 and IL-8 levels, has a relationship with lung functions in obese asthmatic patients.[47] In obese asthmatic patients, FeNO evaluation does not seem to be affected by obesity[48] and can be useful in defining the type of inflammation and the cause of respiratory symptoms. High FeNO levels were found in obese asthmatics with an eosinophilic inflammation, which can be the trigger of bronchial symptoms while low FeNO levels are the result of restrictive rather than obstructive functional changes mainly caused by obesity.[49]

Gastroesophageal reflux disease (GERD) is a common comorbidity of asthma, mildly associated with neutrophilic airway inflammation without impact on FeNO levels[50] in subjects treated with controller medication for asthma. A subgroup of asthmatic patients is complicated by obstructive sleep apnoea that did not seem to impact on FeNO levels.[51] Patients with allergic rhinitis showed evidence of peripheral airway inflammation with increased FeNO levels,[52] impairing the clinical interpretation of FeNO levels.[53] Asthmatic patients with active allergic rhinitis had increased FeNO levels, which decreased together with Asthma Control Test (ACQ) when patients were treated with nasal corticosteroids.[54] Low FeNO levels in uncontrolled moderate/severe asthmatic patients can predict the presence of bronchiectasis.[55]

Factors interfering with FeNO levels

Smoking

Acute and chronic smoking reduces FeNO levels[26,56] suggesting stopping smoking at least one hour before FeNO evaluation.[17] A recent systematic review on FeNO in smoking asthmatic subjects concluded that FeNO levels are decreased in smoking compared to non-smoking subjects but still higher than in smoking controls; however, due to the uncertainty of the published results, caution is needed to interpret FeNO levels in smoking asthmatics.[57]

Viral or bacterial infections

Controversial findings have been published regarding FeNO levels in asthmatics during or after viral/bacterial infections. Infections are frequently the trigger of acute asthma exacerbations; FeNO levels do not seem to distinguish viral from non-viral asthma exacerbation.[58] Malka J et al., found that asthmatic children with exacerbations had lower FeNO levels when PCR for rhinovirus was positive,[59] while asthmatic children with cold like symptoms and negative viral nucleic acid/rhinovirus copies had increased FeNO suggesting airway T2 inflammation due to other causes such as low compliance, high sensitizer exposure, etc.[60] Asthmatic subjects, both young adults and adolescent, without infection symptoms but positive for human rhinovirus do not have

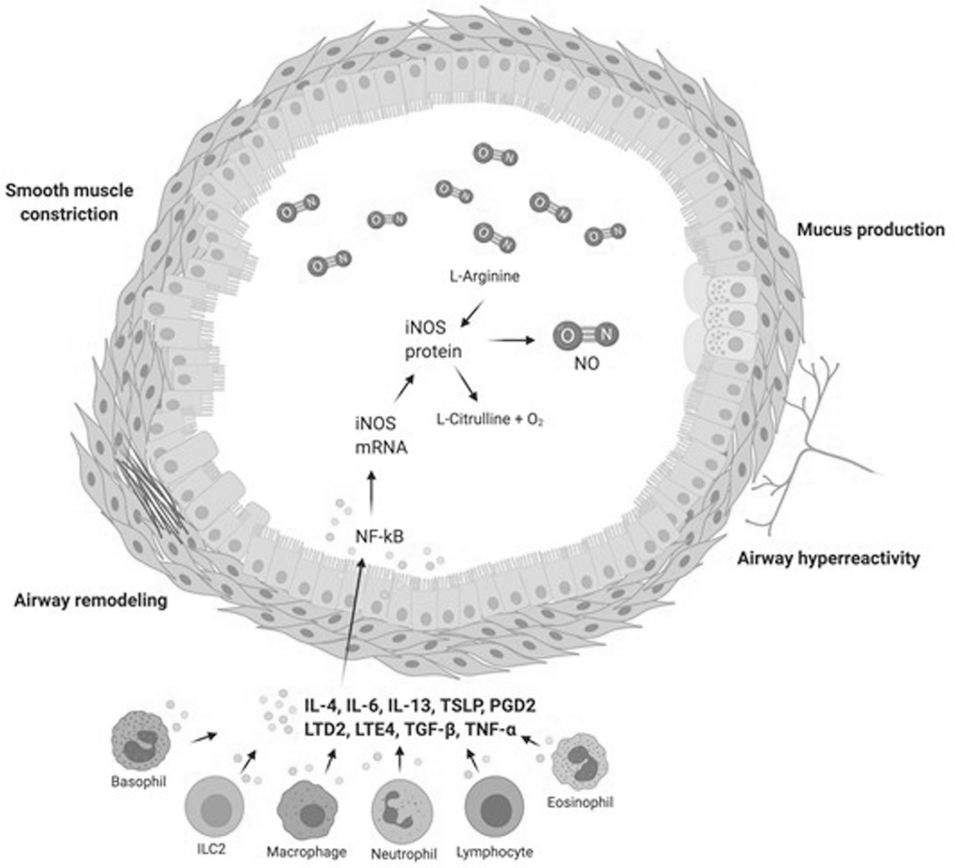

Figure 2 Airways of asthmatic patients are characterized by reversible bronchoconstriction, bronchial hyperresponsiveness and airway remodelling. Nitric oxide (NO) present in the airways is mainly produced by epithelial cells as a consequence of the activation by cytokines of the transcription factor NK-kB which stimulates production of inducible nitric oxide syntetase (iNOS), the enzyme responsible for NO production. Figure was created with Biorender.com.

increased FeNO and blood eosinophils compared to negative asthmatic subjects.[61]

Iikura et al. reported that adult asthmatic patients with viral infections had lower FeNO levels during asthma exacerbation than non-infected subjects,[62] while patients with bacterial infections, mainly due to *S. pneumonia* and *H. influenzae*, had comparable levels of FeNO to non-infected patients both during exacerbation and in stable conditions.[62,63]

Exposure to air pollution

Many studies underlined the association between air pollution and airway inflammation. The relationship between air pollution exposure and FeNO has been mainly studied in children, with a correlation found between ultrafine particle concentrations (<0.1 μm) and FeNO levels in atopic subjects.[64] Unselected children exposed to industrial pollution presented increased odds ratio of having higher FeNO (>30 ppb).[65] Exposure to nitrogen dioxide, as indicator of air pollution, has been associated with FeNO levels in children living in Australian cities, suggesting a possible detrimental inflammatory effect of this compound in subjects with genetic and epigenetic susceptibility of iNOS.[66] Acute exposure to traffic air pollution did not affect FeNO production in adult subjects with mild asthma.[67] FeNO levels were associated with concentrations of $PM_{2.5}$ in unselected older women, some of them with chronic inflammatory airway conditions and exposed to air pollution.[68]

Indoor pollution can also cause/affect airway inflammation. The presence of dampness, moulds, particularly *Aspergillus versicolor* DNA, in schools was associated with high FeNO in students.[69] Recently a correlation between FeNO levels and indoor mycobiome was found in severe asthmatic patients.[70]

Exposure to low doses of Dermatophagoides pteronissinus, was associated with increased FeNO in atopic patients with mild asthma without worsening of symptoms.[71]

Occupational exposure

Work exposure to high or low molecular weight agents can induce, in predisposed subjects, occupational asthma.[72] Moreover, work substances even if not directly responsible for asthma can determine increase in airway inflammation and work exacerbation of pre-existing asthma. FeNO is considered a non-invasive methodology to assess airway inflammation in the diagnostic work-up of occupational asthma.[73] Its use in this context can be useful when combined with specific inhalation challenge, when evaluated at and away from work. Among apprentices, FeNO levels are associated with sensitization and its increase

Table 1 Relationship between FeNO levels and comorbidities in asthmatic patients.

Comorbidity	FeNO	Patients	Advantage of FeNO evaluation	Ref. #
Chronic rhinosinusitis with nasal polyposis	Levels increased in asthmatic patients with chronic rhinosinusitis with nasal polyposis	Severe asthmatics (n = 695); nasal polyposis (n = 282), 2.5% smokers; ICS and OCS treated	To select patients with nasal polyposis even when blood eosinophils are low	43
	Levels associated with nasal polyposis	Severe asthmatics (n = 93); nasal polyposis (n = 28); 12% smokers; all ICS treated, 30% OCS treated		44
Obesity	Levels not affected by increased BMI	Severe asthmatics (n = 286); obese (n = 96); smoking history not reported; most of the patients in corticosteroid treatment	Help in distinguishing when obesity is a comorbidity of asthma or the cause of lung function impairment	48
	High FeNO is associated with more obstructive changes in obese patients	Asthmatics (n = 472); obese (n = 248); current smokers (n = 56); ICS treated (n = 82)		49
Gastroesophageal reflux disease (GERD)	Low FeNO associated with GERD symptoms	Asthmatics (n = 248), smoking history not reported, treated with ICS (n = 246); proton pump inhibitory therapy (n = 61).	Presence of high FeNO in GERD patients suggests eosinophilic inflammation due to other causes	50
Obstructive Sleep Apnoea Syndrome (OSAS)	Levels not affected by OSAS	ICS treated asthmatics (n = 60), Smoking history not reported	Presence of high FeNO in OSAS patients suggests eosinophilic inflammation due to other causes	51
Allergic rhinitis	FeNO increases with active allergic rhinitis, nasal steroid treatment decreases FeNO levels	Asthmatics (n = 520); with allergic rhinitis (n = 348), No current smokers, 397 never smokers. Nasal mometasone furoate treated (n = 40)	In asthmatics with allergic rhinitis high FeNO alerts to uncontrolled nasal symptoms	52
Bronchiectasis	Low FeNO was associated with presence of bronchiectasis	Uncontrolled moderate to severe asthmatics (n = 398); with bronchiectasis (n = 113), non smokers, ICS treated	Higher FeNO levels (> 20.5 ppb) suggest lower probability of bronchiectasis	55

ICS = inhaled corticosteroid therapy, OCS = oral corticosteroid therapy.

after work exposure related to the incidence of bronchial hyperreactivity.[74–76]

Physical exercise

Physical exercise can affect symptoms and airflow limitation in asthmatic patients. FeNO levels decreased after acute exercise of moderate intensity particularly at low temperatures in asthmatic or allergic patients.[77] FeNO does not predict a positive exercise test in children with respiratory symptoms during physical activity.[78] Exercise, such as aerobic training, in patients with moderate or severe persistent asthma can reduce FeNO and sputum eosinophils in

patients with worse airway inflammation.[79] As to the cumulative effect of swimming activity, Škrgat et al. showed that FeNO and blood eosinophils did not differ between intensive training and stopping period in non-asthmatic adult competitive swimmers.[80] The decrease of FeNO, as acute effect, reported hours after the swimming activity has been hypothesized to be caused by either a direct neurogenic response or an inhibitory effect on iNOS.[81]

FeNO versus other biomarkers of T2 inflammation

FeNO levels slightly correlated with airway eosinophils evaluated through induced sputum,[82] bronchoalveolar lavage[83] while contrasting results were found with biopsies.[32–84]

Most of FeNO in the airways is produced by iNOS stimulated by IL-4/IL-13 signalling during inflammatory processes. T2 inflammation in asthma is characterized by an increase of different cytokines reflecting the activation of specific immunologic pathways; FeNO levels seem to correlate better with IL-4 and IL-13 while eosinophils with IL-5. IL-13 is one of the main drivers of iNOS activation and consequent FeNO production.[85] In the meantime, IL-13 activates eosinophils and favours eosinophil extravasation through the up-regulation of adhesion molecules in the endothelium. Furthermore, IL-13 increases in the airways the production of chemokines able to bind the CCR3 receptor and chemotactic for eosinophils.[86]

FeNO and blood eosinophils can be used independently to predict airway inflammation; they did not correlate each other in uncontrolled asthma[87] and mildly correlated in non-smoker asthmatics.[88] A weak correlation between blood eosinophils and FeNO levels was found in young asthmatic patients, and subjects with simultaneous increase of FeNO and blood eosinophils had higher bronchial hyperreactivity and low asthma control.[29] However, discrepancies between FeNO levels and blood eosinophils have been reported in subgroups of asthmatic patients.[89] In non-smokers with mild-moderate persistent asthma, FeNO had high sensitivity in predicting eosinophilic asthma compared to blood eosinophils and serum eosinophil cationic protein (ECP).[90] Subjects with high FeNO and low blood eosinophils had higher numbers of sensitization to inhaled allergens than subjects with low FeNO.[91] FeNO as single measurement, failed to predict persistent blood eosinophilia in patients with new-onset asthma compared to single blood eosinophil evaluation.[92] Blood eosinophils have higher sensitivity for sputum eosinophilic inflammation than FeNO and ECP in asthmatic patients treated with inhaled corticosteroids.[93] Considering recent findings on different mechanisms driving eosinophilic inflammation and FeNO, it is not surprising that blood eosinophils prove more sensitive as surrogate marker of sputum eosinophils than FeNO in moderate-severe asthmatic patients.[85]

FeNO and total serum IgE are elevated in asthmatic patients, particularly in the atopic ones and they weakly correlate.[94] FeNO was reported superior to total IgE and equal to blood eosinophils in predicting sputum eosinophilia in different subset of patients with adult onset asthma, independently to smoking history, atopy or disease severity.[95]

Periostin, an extracellular matrix protein, reflects activation of T2 mechanisms.[96] In a study of asthmatic patients treated with the anti IL-13 lebrikizumab, subjects with high serum periostin showed improvement in lung functions compared to patients with low levels[97] but these results were not confirmed in subsequent studies.[98] FeNO and periostin can be simultaneously increased in asthmatic patients and in this case, they can identify severe T2/eosinophilic airway inflammation.[99] When FeNO was used together with periostin and peripheral blood eosinophils to ameliorate the sensitivity of biomarkers, the combination failed to increase the predictive value for asthma exacerbations.[22]

In a recent analysis from the U-BIOPRED study group, serum periostin failed to predict a T2 response of airway epithelial cells compared to FeNO, blood and sputum eosinophils in asthmatic patients.[11] During acute severe exacerbations, T2 biomarkers peak but mechanisms, which induce their increase are differently sensitive to steroid therapy. Semprini R. et al., demonstrated that in asthmatic patients evaluated after treatment for severe exacerbations, FeNO decreased 2 weeks, serum periostin 1 week and blood eosinophils 1 day after oral steroid intake.[100] In symptomatic patients despite maximal ICS dose, serum periostin was the best predictor of airway eosinophilia compared to FeNO and blood eosinophils.[101] In smokers with less controlled asthma, blood eosinophil evaluation is the most accurate in predicting airway eosinophilic inflammation compared to FeNO, IgE, serum periostin and IL-13.[102]

A recent position paper of the European Academy of Allergy and Clinical Immunology evaluating the clinically applicable biomarkers for asthma, recognized FeNO as the best point-of care biomarker to identify T2 endotype in asthma.[103] Table 2 summarizes the relationship between FeNO and other biomarkers of T2 inflammation when used to predict high airway eosinophils.

FeNO and therapeutic strategies

ICS therapy

Inhaled corticosteroid therapy affects NO production by iNOS, reducing FeNO levels in treated asthmatic patients.[104] For this reason, FeNO evaluation has been proposed to monitor disease control in asthmatic patients characterized by eosinophilic inflammatory phenotype at diagnosis. A 20% fall in FeNO or 10 ppb has been proposed as a significant response to inhaled corticosteroid treatment when starting values are >50 ppb or <50 ppb respectively.[105] FeNO levels decrease two weeks after severe asthma exacerbations treated with systemic corticosteroids, and return to stable levels after 4–8 weeks.[100] In patients with more than one exacerbation/year, treatment guided according to FeNO levels seems useful in maintaining disease stability.[106] A systematic review and meta-analysis on sputum and FeNO guided treatment in asthmatic subjects confirmed the reduction of exacerbations in patients monitored with these biomarkers but disease control and lung function were similar to those patients followed in a traditional way.[107] The same was found in patients with mild/moderate persistent asthma.[108] FeNO proved useful in predicting response to ICS in patients with non-specific respiratory symptoms and lack of bronchodilator reversibility.[109] In stable asthma, basal and serial evaluation of FeNO is not able to predict treatment failure

Table 2 Accuracy of different T2 biomarkers in reflecting sputum eosinophila considered as sputum eosinophils higher than 3.0%, 2.5% or 2.0%.

Ref.#	Airway eosinophilic inflammation (sputum eos)	Asthmatic Patients	Smokers	ICS	FeNO	Blood eosinophils	Serum periostin	Serum Total IgE	Serum ECP
[87]	2.5%	Adult uncontrolled (n = 75)	yes	yes	0.71(67.3–37.9)	0.73 (61.5–78.3)	ND	ND	ND
					AUC (sens %-spec %)	AUC (sens %-spec %)			
[90]	3.0 %	Adult mild or moderate (n = 124)	no	no	0.85 (0.74–0.97) AUC (95% CI)	0.92 (0.84–0.99) AUC (95% CI)	ND	ND	0.75 (0.87–1.0) AUC (95% CI)
			no	yes	0.73 (0.55–0.91) AUC (95% CI)	0.70 (0.47–0.93) AUC (95% CI)	ND	ND	0.51 (0.27–0.76) AUC (95% CI)
[93]	3.0%	Adult moderate, severe External validation (n = 110)	no	yes	0.78 (0.66–0.89) AUC (95% CI)	0.89 (0.81–0.96) AUC (95% CI)	0.55 (0.43–0.67) AUC (95% CI) Non-significant	ND	ND
	2.0%	Replication cohort (n = 37)			0.79 (NR) AUC (95% CI)	0.88 (NR) AUC (95% CI)	NR	ND	ND
[95]	3.0 %	Adult-onset (n = 336)	yes	yes	0.82 (0.77–0.87) AUC (95% CI)	0.83 (0.78–0.87) AUC (95% CI)	ND	0.69 (0.63–0.75) AUC (95% CI)	ND
[101]	3% + biopsy eos. $\geq 22/m^2$	Adult symptomatic (n = 67)	no	yes	0.79 (NR)	0.71 (NR)	0.84 (NR)	0.62 (NR)	ND
					AUC (95% CI	AUC (95% CI	AUC (95% CI	AUC (95% CI	
[102]	3%	Adult with loss of disease control (n = 47)	yes	yes	0.76 (0.65-0.810.65–0.81)	0.92 (0.85–0.97)	0.56 (0.46–0.66)	0.51 (0.41–0.61)	ND
					AUC (95% CI)	AUC (95% CI)	AUC (95% CI)	AUC (95% CI)	

Ref.#= reference number; eos = eosinophils; AUC = Area Under the Curve; CI = confidence interval; ICS = inhaled corticosteroid treatment; ND = not done, NR = not reported.

confirming that this biomarker alone should not be recommended for this purpose.[110]

Recent results of a multicentre randomized control trial in patients with mild asthma showed that only in patients with blood eosinophil count greater than 0.3×10^9/L maintenance with budesonide plus as needed salbutamol was efficacious in decreasing exacerbations while FeNO or composite scores are not useful for this purpose.[111]

FeNO may help evaluate disease stability in pregnant asthmatic women, since during pregnancy, asthmatic patients monitored with FeNO reduced exacerbations and increased quality of life.[112] Cough is a non-specific symptom of asthma in common with other conditions, sensitive to ICS treatment when caused by inflammation. FeNO has been proposed to monitor patients with corticosteroid responsive cough.[113,114]

FeNO as a predictor of target treatment response and effects of these therapies on FeNO levels

FeNO, together with other T2 biomarkers, has been used to monitor the efficacy of target therapies with monoclonal antibodies in severe asthmatic patients. Asthmatic patients with high FeNO ($\geq$ 19.5 ppb) decrease the number of exacerbations when treated with omalizumab more than subjects with low FeNO (<19.5 ppb).[115] Recent ERS/ATS task force for severe asthma management, concluded that FeNO, together with blood eosinophils, can identify which patients are more responsive to omalizumab in terms of reduction of exacerbations and impairment of lung functions, but more studies are needed to evaluate other outcomes.[116] FeNO and blood periostin seem the best markers for selecting asthmatic patients to be treated with tralokinumab or with lebrikizumab, two anti IL-13 monoclonal antibodies.[117,118] Treatment of severe asthmatic patients with dupilumab, an antibody to the α subunit of the IL-4 receptor, reduced disease exacerbations and FeNO levels, both in allergic and in non allergic patients.[119] Furthermore, FeNO levels $\geq$ 25 ppb in severe asthmatic patients predict significant response to dupilumab.[120]

Not all target therapies aimed to inhibit T2 inflammation affect FeNO levels. FeNO is not reduced by anti-IL-5 monoclonal antibodies, which efficiently reduce eosinophilic inflammation but have limited effect on epithelial cell activation.[121] Real world studies confirmed the efficacy of mepolizumab treatment without affecting FeNO levels.[122]

FeNO and blood eosinophils were not reduced by administration of fevipiprant, an oral prostaglandin D_2 receptor 2 antagonist that was successful in reducing sputum eosinophils in patients with moderate/severe asthma and persistent high sputum eosinophils despite treatment with inhaled corticosteroids.[123]

Treatment targeting epithelial-cell–derived cytokine thymic stromal lymphopoietin (TSLP) resulted in a significant decrease of FeNO together with asthma exacerbations both in eosinophilic and in non-eosinophilic asthmatic subjects, suggesting the strict relationship between TSLP and FeNO production.[124] Recently a mixed inflammatory pattern characterized by severe T2 and T17 high has been described. In this study, asthmatic patients with elevated just FeNO or with both elevated FeNO and blood eosinophils have increased IL-5, IL-8, IL-13 and IL-17A, which can cause a massive tissue destruction and consequently a more severe clinical phenotype,[89] opening the possibility that FeNO can identify patients to target other molecules.

Table 3 summarises the effects of target therapies on FeNO and other biomarkers.

Rehabilitation programmes

Asthma and particularly severe asthma can benefit from rehabilitation programs.[131] However, bronchial inflammation, at least type 2, does not seem to be decreased by breathing retraining programs, which instead have effects on asthma related quality of life.[132] The same is true for high-intensity interval training performed by non-obese asthmatic patients which had no effect on airway inflammation evaluated with FeNO while a significant improvement in asthma control and quality of life was reported.[133] FeNO also remained unchanged after 4 week therapist-led sessions to manage disease while asthma control increased.[134] Aerobic training reduced FeNO and sputum eosinophils in asthmatic patients with higher airway inflammation at baseline.[78]

Clinical implications

FeNO measurements determined changes in therapeutic programs in about 30% of the cases and in 90% when corticosteroid treatment was considered.[135] Results of a real-world survey highlighted that airway inflammation is often underestimated by clinicians with respect to FeNO determination and its evaluation could be added to other measures to achieve asthma control.[135] Expert committee of the Global Initiative for Asthma (GINA) document reported that having high FeNO is a risk factor for exacerbations in allergic asthmatics taking ICS. They also reported that, due to lack of long-term studies, FeNO cannot be recommended to step down ICS therapy, however FeNO guided treatment was recognized as evidence A in reducing exacerbations in children.[1]

Applicability to low income countries

Asthma is considered to be one of the chronic respiratory conditions, affecting the Disability-Adjusted Life Years index, which evaluates the years of life spent with disabilities.[136] In many low income countries, prevention, diagnosis and management of asthma is quite difficult due to low perception of risk factors (indoor and outdoor exposures), low availability of diagnostic measures/devices, low adherence and use of alternative medicine, low possibility of follow-up,[137,138] and difficulties in recovering high cost therapies for severe asthmatic patients. FeNO is an exhaled biomarker, which is easily detectable by on-line and off-line devices, frequently measured in children, in whom other inflammatory biomarkers have limited use. Measures can be easily performed by minimally trained personal, particularly for portable devices. This facilitates its spread in research and clinical settings, making the evaluation possible in many countries, including the less developed. FeNO was used to characterize severity of East African asthmatics, highlighting the reduced access to therapy in this area of the world.[139] In rural zones of South Africa FeNO measurements have been used to assess airway inflammation in women exposed

Table 3 Variation of T2 biomarkers after target therapy in patients with severe asthma.

Target therapy	MoAb	FeNO	Blood eosinophils	Serum periostin	Sputum eosinophils	Ref. #
Anti IgE	Omalizumab	ND	↓	ND	↓	125
		↓	↓	ND	↓	115
Anti IL-5/IL-5R	Mepolizumab	=	↓↓	ND	↓	121
	Reslizumab	ND	↓	ND	↓	126
		↓	↓	ND	ND	127
	Benralizumab	ND	↓↓	ND	↓↓	128
Anti IL-13	Lebrikizumab	↓	=	↓	ND	129
	Tralokinumab	↓	=	=	=	117
Anti IL-4Rα	Dupilumab	↓	↓	↓	ND	119
Anti TSLP	Tezepelumab	↓	↓	ND	↓	130
Antagonist of prostaglandin D2	Fevipiprant	=	=	ND	↓	123

MoAb = Monoclonal Antibody; Ref.#= reference number; IL-= Interleukin-; IL-5R = Interleukin-5 Receptor; TSLP = Thymic stromal lymphopoietin; = unchanged, ND = not done.

to pesticides.[140] FeNO and sensitization state were evaluated in severe asthmatic children living at high altitude in Bogotá.[141] Exposure to paraffin used for cooking in low socio-economic communities in Africa, was associated with increased FeNO levels and rhinitis symptoms in children.[142] FeNO levels evaluated in Malaysian office workers were associated with atopy and respiratory symptoms and with the amount of sieved dust even when analysis was corrected for indoor temperature or relative air humidity.[143,144]

Published studies conducted in low income countries showed that FeNO evaluation might be useful in different settings to monitor asthmatic patients and to evaluate impact of different exposures on airway inflammation. Cost effectiveness studies on FeNO used both at diagnosis and during asthma monitoring in different countries,[145–147] showed that FeNO can reduce the cost of disease management particularly in severe patients. This economic evaluation could also favour FeNO use in low-income countries, but more studies in this setting, particularly focused on costs and outcomes, are needed.

Research priorities

As proposed by GINA panel of experts, long follow-up studies focused on step-down ICS therapy based on low FeNO levels are needed.[1] Furthermore, considering the utility of this biomarker to define T2 inflammation, large cohort studies in low income countries might reveal its usefulness in asthma prevention and disease control.

Conclusions

FeNO production by iNOS in asthma can vary and be associated with different disease markers. Many factors and interventional approaches can influence FeNO levels and should be considered in clinical settings and in follow-up studies in order to correctly interpret FeNO levels. Additional biomarkers have been recognized as T2 sign marks, and due to the complexity of the immunological pathways, they are only partially overlapping. ICS therapy significantly affects FeNO levels but target therapies with monoclonal antibodies impact on FeNO levels only when the target molecule interplays with mechanisms involved in FeNO production. FeNO should be considered a useful aid in low income countries to monitor asthmatic patients and to survey their exposure to possible inflammatory agents.

Authors' contributions

PP, DV, SL, AGM, JWCA and AS conceived and drafted the manuscript with the support of GBM. AGM prepared figures. All authors critically reviewed and edited the final version prior to submission.

Conflicts of interest

The authors have no conflicts of interest to declare.

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LETTER TO THE EDITOR

Outcomes of COVID-19 patients treated with noninvasive respiratory support outside-ICU setting: a Portuguese reality

To the Editor

Noninvasive respiratory support (NRS) has become a valuable tool in COVID-19 acute respiratory failure (ARF) management. However, there is a lack of consensus in for or against its use due to the risks of intubation delay and aerosols environmental contamination.[1] Strategies to mitigate NRS iatrogenic risks and healthcare workers infection have been suggested.[2,3]

Despite efforts to increase ICU resources, to cope with patient influx, general wards worldwide were converted in respiratory intermediate care units (RICU), where patients in need for NRS were treated.[4,5] Our goal was to evaluate the Portuguese reality regarding safeness and outcomes of NRS in COVID-19 ARF in a non-ICU setting.

Adult patients with COVID-19 pneumonia needing NRS [high flow nasal cannula (HFNC), continuous positive airway pressure (CPAP) and/or noninvasive ventilation (NIV)], treated in a RICU, were prospectively enrolled from November 18th, 2020 to February 18th, 2021. Institutional review boards authorised the study (n. 22/2021). Informed consent was waived.

High flow nasal cannula was instituted if arterial oxygen partial pressure to fractional inspired oxygen (PaO2/FiO2) $\leq$ 200 mmHg with FiO2 $>$ 0.4, respiratory rate (RR) $\geq$ 25cpm and/or sings of respiratory distress. Continuous positive airway pressure and NIV was started if SpO2 $<$92%, RR $\geq$ 25bpm and/or sings of respiratory distress in patients with HFNC failure or *ad initium* if HFNC failure was expected. Arterial oxygen partial pressure to fractional inspired oxygen ratio was accessed before NRS institution. In patients with HFNC failure who increased support to CPAP/NIV, the PaO2/FiO2 considered was the one before CPAP/NIV. Noninvasive respiratory support settings were adjusted to maintain SpO2 92−96%. Continuous positive airway pressure and NIV was delivered by oronasal mask. All patients had a "do intubate" (*DI*) or "do not intubate" (*DNI*) order defined at admission. Baseline patient's characteristics were compared according to NRS technique (HFNC vs. CPAP/NIV). The success rate was defined by invasive mechanical ventilation (IMV)-free survival. Several outcomes were recorded: length of stay, complications, need for endotracheal intubation (ETI) and mortality. The association between NRS and outcomes was calculated using a logistic regression model adjusted for age, *DNI* order and PaO2/FiO2. A two-sided test of $<$0.05 was considered statistically significant.

190 out of 1748 hospitalized patients were treated with NRS in RICU. Table 1 summarizes patient's characteristics and treatment.

50 patients needed ICU admission, 25.2% in HFNC group and 29.4% in CPAP/NIV group ($p = 0.366$). Considering only *DI* patients, 42.0% were escalated to ICU. Time from NRS to IMV was 3.5 $\pm$ 3.3 and 3.9 $\pm$ 2.8 days in survivors and deceased patients, respectively (p = 0.724). In-RICU and in-hospital mortality were 24.2% and 36.3%, respectively. In *DI* patients, there were no deaths in RICU and in-hospital mortality was 16.0%. In *DNI* patients, mortality rate was 50.0% and 58.9% in-RICU and in-hospital, correspondingly. Globally, 63.7% survived hospitalization, 53.2% exclusively treated with NRS techniques in RICU.

Table 2 illustrates outcomes according to NRS. In HFNC group, 61.9% were successful weaned and 27.1% needed ETI. The success rate in *DI* and *DNI* patients was 68.2% and 51.9%, respectively ($p < 0.001$). In-hospital mortality was 27.3% (14.1% for *DI vs*. 48.1% for *DNI* patients, $p < 0.001$). In CPAP/NIV group, CPAP was the preferred technique (56.9%). 29.4% were successfully weaned and 53.3% needed ETI. The success rate in *DI* and *DNI patients* was 40.0% and 25.0%, respectively ($p = 0.005$). In-hospital mortality was 60.8% (26.7% in *DI vs*. 75.0% in *DNI* patients, $p = 0.001$).

During the study period, our hospital received 10% of national cases of COVID-19. 10.9% were treated in RICU and 50% were successfully managed with NRS.

Considering patients with *DI order*, ICU admission was avoided in almost 60% and only a third needed ETI. Delays in ETI have prognostic impact and it has been suggested that NRS might have contributed to the problem. Time to IMV was similar in survivors and deceased patients. Moreover, casualties in RICU were not reported and in-hospital mortality was lower than previously described[5,6], supporting strategy safeness.

In-hospital mortality was 36.6%, higher than in other series.[5,7,8] It is important to note that, in our sample, ARF was severe and almost 50% were *DNI* patients. Interestingly, 41% of *DNI* patients survived hospitalization supporting the role of NRS in patients with therapeutic ceiling.

We report a success rate of 62% in HFNC group, with a survival greater than 50% in *DNI* patients. In CPAP/NIV treatment group, success was achieved in 30%. Patients in this

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Table 1 Demographic and clinical characteristics of the study population and according to cohort.

	Total	Cohort		p-value
		HFNC	CPAP/NIV	
Patients	190 (100)	139 (73.2)	51 (26.8)	
Age (years)	66.7 (11.8)	65.7 (12.2)	69.6 (10.2)	0.043
Male gender	130 (68.4)	95 (68.3)	35 (68.6)	0.970
Do not intubate order	90 (47.4)	54 (38.8)	36 (70.6)	<0.001
Comorbidities				
Charlson comorbidity index (points)	3.3 (2.0)	3.2 (2.1)	3.6 (1.7)	0.271
Hypertension	127 (66.8)	87 (62.6)	40 (78.4)	0.026
Dyslipidemia	122 (64.2)	92 (66.2)	30 (58.8)	0.380
Body mass index (Kg/m^2)	28.6 (5.9)	28.2 (5.7)	29.5 (6.2)	0.174
Diabetes	65 (34.2)	47 (33.8)	18 (35.3)	0.849
Heart failure	30 (15.8)	19 (13.7)	11 (21.6)	0.186
Stroke	14 (7.4)	11(7.9)	3 (5.9)	0.763
Chronic kidney disease	12 (6.3)	8 (5.8)	4 (7.8)	0.736
COPD	11 (5.8)	8 (5.8)	3 (5.9)	1.000
Length of symptoms (days)	8.1 (3.7)	7.9 (3.8)	8.6 (3.3)	0.288
Laboratory findings				
PaO2/FiO2 (mmHg)	98.3 (67.5)	108.9 (76.3)	70.9 (18.1)	<0.001
Lymphocytes count (10^9 cells per L)	0.7 (0.7)	0.7 (0.8)	0.7 (0.5)	0.526
Platelet count (10^9 cells per L)	185.2 (72.1)	184.6 (71.3)	186.8 (74.7)	0.850
C-reactive protein (mg/L)	191.0 (95.6)	183.5 (88.7)	211.5 (110.6)	0.073
Procalcitonin (ng/mL)	3.3 (15.3)	2.2 (7.1)	6.4 (27.5)	0.103
Ferritin>1500 (ng/mL)	72 (40.0)	54 (38.9)	18 (35.3)	0.680
Lactate dehydrogenase (U/L)	503.6 (234.5)	465.1 (191.9)	609.7 (302.1)	<0.001
Interleukin-6 (pg/mL)	238.2 (652.3)	199.3 (591.2)	350.5 (800.1)	0.176
D-dimer (μg/mL)	4876.0 (10374.2)	4043.1 (9235.0)	7221.5 (12884.5)	0.065
Treatment				
Steroids	185 (97.4)	134 (96.4)	51 (100.0)	0.170
Remdesivir	29 (15.3)	28 (20.1)	1 (2.0)	0.001
NRS parameters and mode				
Maximum FiO2		0.9 (0.1)	1.0 (0.1)	-
Maximum flow (L)		59.2 (1.0)	-	-
Maximum CPAP/EPAP (cmH2O)		-	10.0 (1.9)	-
Maximum IPAP (cmH2O)		-	14.8 (2.4)	-
Length of treatment (days)		5.5 (4.4)	5.2 (4.3)	-

Data are presented as number (percentages) or mean (standard deviation) as appropriate. HFNC: high flow nasal cannula; CPAP: continuous positive airway pressure; NIV: noninvasive ventilation; COPD: Chronic obstructive pulmonary disease; PaO2/FiO2: arterial oxygen tension /inspiratory oxygen fraction. NRS: noninvasive respiratory support. EPAP: expiratory positive airway pressure. IPAP: inspiratory positive airway pressure.

group were older, had more severe ARF and 71% were *DNI*. Mortality rates in patients treated outside-ICU, in whom NIV was the therapeutic ceiling, reached 76% in previously reports, which is in line with our findings.[6,7,9] Nevertheless, CPAP/NIV prevented IMV in 40% of the candidates, with mortality rates similar to published data.[10]

The difference in ETI and mortality rates between groups disappeared after adjustment for confounders, emphasising that outcomes don't rely on the NRS technique. Complications were infrequent. Pneumothorax/pneumomediastinum is reported in 2% of ICU treated patients.[11] Barotrauma occurred in both groups equally after adjustment for confounders, suggesting that underling disease severity might play a role.

Our study has limitations: the decision to start on a specific NRS technique greatly relied on equipment availability and is single-centered with a limited sample.

To the best of our knowledge this is the first prospective study on NRS outside-ICU in COVID-19 pneumonia, conducted in one of the Portuguese regions that was most affected. Both HFNC and CPAP/NIV seem to be equally feasible and outside-ICU should be considered for selected patients in resource-constrained settings.

Contributions of the authors

Lígia Rodrigues Santos, Rafaela Gonçalves Lopes, Ana Silva Rocha, Marta Dalila Martins, Teresa C. Guimarães, Mariana Meireles and Helena Vilaça collected the clinical and biological data from the files.

Mariana Meireles and Helena Vilaça, provided assistance for statistical analysis.

Lígia Rodrigues Santos, Rafaela Gonçalves Lopes, Mariana Meireles and Helena Vilaça wrote the manuscript.

Alice Castro, Mari Mesquita revised the manuscript

All the authors have checked the manuscript.

Table 2 Clinical outcomes and relative probability according to noninvasive respiratory support technique.

	Total	HFNC	CPAP/NIV	OR (95% CI)	p-value
Length of stay (days)	15.2 (13.0)	15.4 (13.6)	14.7 (11.3)	0.99 (0.97-1.02)	0.740
Technique complications					
Facial ulcers	3 (5.9)	-	3 (5.9)	-	-
Intolerance	3 (5.9)	0 (0.0)	3 (5.9)	-	-
Aspiration	0 (0.0)	-	0 (0.0)	-	-
Pneumothorax | Pneumomediastinum	7 (3.7)	3 (2.6)	4 (7.8)	-	-
Crude				3.86 (0.833-17.88)	0.084
Adjusted[#]				1.76 (0.29-10.84)	0.541
ETI	31 (31.0)	23 (27.1)	8 (53.3)		
Crude				3.08 (1.00-9.46)	0.049
Adjusted[¶]				1.82 (0.47-7.02)	0.388
Hospital mortality	69 (36.3)	38 (27.3)	31 (60.8)		
Crude				4.12 (2.10-8.09)	<0.001
Adjusted[#]				1.27 (0.27-5.97)	0.765

Data are presented as number (percentages) or mean (standard deviation) as appropriate. HFNC: high flow nasal cannula; CPAP: continuous positive airway pressure; NIV: noninvasive ventilation; ETI: endotracheal intubation.
[#] adjusted for age, arterial oxygen tension/inspiratory oxygen fraction ratio and "do not intubate" order.
[¶] adjusted for age and arterial oxygen tension/inspiratory oxygen fraction ratio.

Conflicts of interest

The authors declare that they have no conflict of interests.

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LETTER TO THE EDITOR

SARS-CoV-2 as a trigger of eosinophilic pneumonia

To the Editor

Chronic eosinophilic pneumonia (CEP) is a rare disease that is most common in women, typically non-smokers and with peak incidence in the third and fourth decades. Asthma precedes the onset of this disease by several weeks to years in up to two-thirds of the cases. CEP presents as a subacute onset of dyspnea and cough associated with systemic symptoms.[1] Wheezing and crackles may be present at auscultation, and in many cases, mild hypoxemia can be detected.[2] The diagnostic criteria consist of the presence of respiratory symptoms for at least two weeks: (1) a typical computer tomography (CT) scan of the chest with diffuse consolidation and/or ground-glass opacities with peripheral predominance, (2) alveolar eosinophilia commonly $\geq$ 40% in the bronchoalveolar lavage (BAL), (3) blood eosinophilia $\geq$ 1000/mm^3, (4) and/or eosinophil infiltration in the lungs in the absence of an alternative diagnosis.[2,3] The standard treatment consists of corticosteroids, and the response to these drugs is usually dramatic.[1,4]

In this report, we describe a case of eosinophilic pneumonia triggered by a severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2/COVID-2019) infection. A 51-year-old male, non-smoking insurance agent with a history of untreated dyslipidemia and uncontrolled asthma for the last year and a half was treated with formoterol and budesonide. The patient presented with myalgia, fever, and cough after having contact with a COVID-19 patient. The diagnosis of COVID-19 was confirmed by reverse transcription-polymerase chain reaction (rt-PCR). After one week at home the patient was asymptomatic, and during the quarantine time, he was only treated with acetaminophen as needed.

Two weeks later the patient started with progressive dyspnea (modified Medical Research Council [mMRC] scale: grade 2), wheezing, and productive cough. In the emergency department, he presented with fever (38 °C) and wheezing. A CT scan of the chest showed ground-glass opacities and crazy paving areas with < 10% lung involvement. At the time, these features were thought to be associated with the previous SARS-CoV-2 infection. The blood analysis showed leukocytosis 14,400/uL (normal 4000—11,000/uL, without blood cell differential) and increased C-reactive protein (CPR) 54.40 mg/L (normal < 0.5 mg/L). Given the possibility of bacterial infection, he was discharged home with cefixime for one week and azithromycin for three days.

After one week of antibiotic treatment, the patient did not feel better and returned twice to the emergency department. He denied a history of weight loss, anorexia, rhinosinusitis, paresthesia, sensibility changes, and skin lesions. The patient also denied consumption of illicit drugs or exposure to toxins. A CT pulmonary angiography was performed that excluded pulmonary embolism but showed worsening consolidations, ground-glass opacities, and crazy paving areas, predominantly peripheral, with about 15% of pulmonary involvement. The arterial blood analysis showed hypoxemia (pO2 60 mmHg) and blood analysis leukocytosis 17160/uL. At the last visit a white blood cell differential was performed showing 51.5% of eosinophils (8840/uL), confirmed by peripheral blood smear. The IgE level was 156 Ui/mL. He started empiric prednisolone 40 mg per day and was admitted to a ward for further diagnostic examination.

After one week, the CT scan showed migratory bilateral consolidations (Fig. 1). The immunological study and blood cultures were negative. Bronchoscopic evaluation of the airways was normal, and the immunologic study of BAL showed increased cellularity with eosinophilia of 94% and normal lymphocyte and neutrophil counts without microbiological isolates and negative cytology. Bilateral nasal polyps were found, and a biopsy confirmed it to be inflammatory lesions of chronic rhinosinusitis without granulomas, vasculitis, or signs of malignancy.

Given these findings, we assumed a diagnosis of eosinophilic pneumonia. The prednisolone dose was increased to 1 mg/kg per day with rapid clinical improvement. Two days later the patient was taken off oxygen and blood eosinophils levels were down to 0.1%. He was discharged home and started follow-up at an interstitial lung disease outpatient appointment. One month later the patient underwent a pulmonary function test that was normal, and a CT scan four months later showed complete resolution of the lung opacities (Fig. 2).

In this report, we describe a rare case of an eosinophilic pneumonia that was triggered by a SARS-CoV-2 infection. To the authors' knowledge, only two cases of acute eosinophilic pneumonia associated with SARS-CoV-2 have been described

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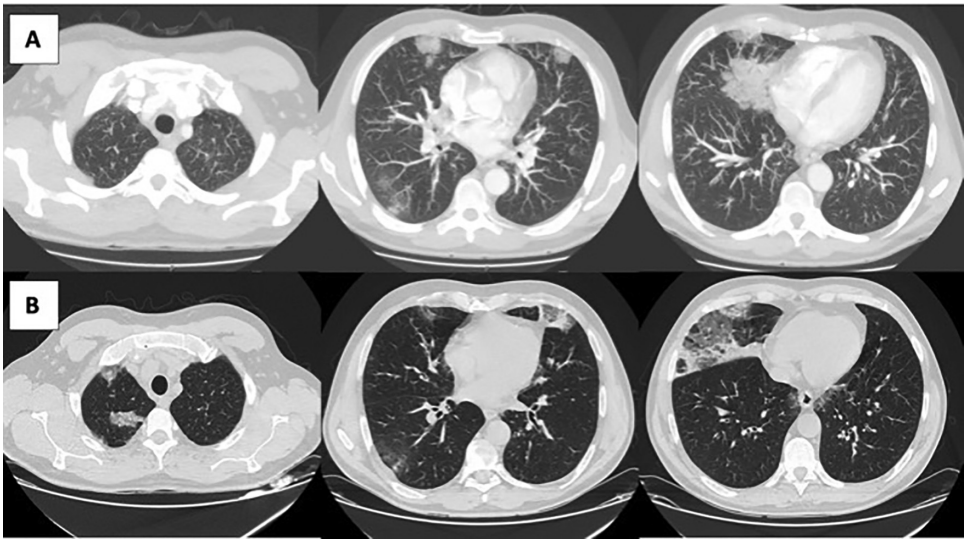

Fig. 1 Imaging evolution of the patient. **A)** Computed tomography (CT) pulmonary angiography with consolidations and ground-glass opacities predominantly peripheral. **B)** CT scan of the thorax taken one week later, showing migratory bilateral consolidations that were predominantly peripheral and an increase in crazy paving areas and ground-glass opacities.

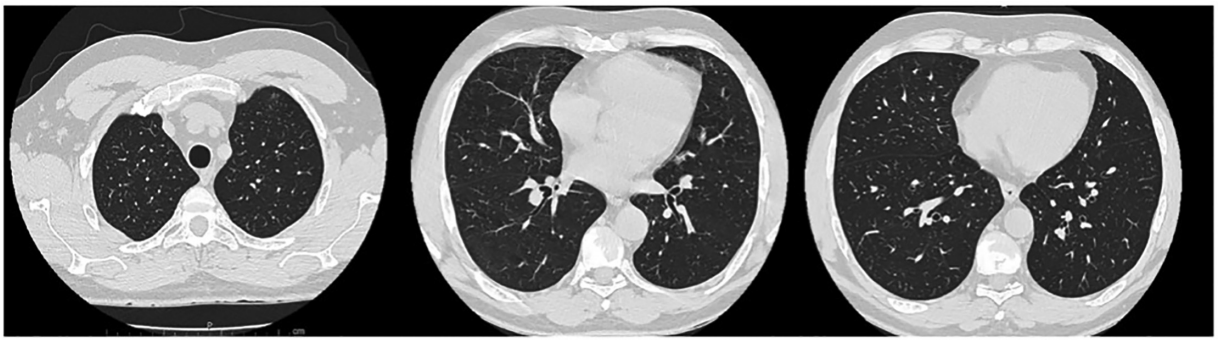

Fig. 2 CT scan, four months after the onset of disease, showing complete resolution of the lung opacities.

in the literature.[5,6] In our case, the patient presented a subacute onset of symptoms, initially presenting three weeks after the COVID-19 diagnosis with lung opacities and blood eosinophilia and then confirmed BAL eosinophilia. This patient was not taking any chronic medication and was not exposed to smoking, illicit drugs, or toxins and showed a dramatic response to corticosteroid treatment. This clinical case supports the already current evidence in which it is important to consider the development of interstitial lung disease in patients with COVID-19 including eosinophilic pneumonia. Despite the absence until now of a known mechanism for eosinophilic pneumonia in SARS-CoV-2, we believe that just as with other viruses, SARS-CoV-2 in rare cases can trigger an eosinophilic response.[7]

Patient's consent

Written informed consent was obtained for the publication of this case report and accompanying images.

Conflicts of interest

The authors have no conflict of interest to declare.

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LETTER TO THE EDITOR

Cycling biologic therapy for severe asthma

To the Editor

Systemic steroids in patients with asthma can induce various complications such as infection, diabetes, and osteoporosis, and increase healthcare resource utilization. Biologics are a corticosteroid-sparing strategy,[1] and recently, "super-responders" i.e., patients who show excellent response to biologics, which results in complete cessation of exacerbations and permits discontinuation of systemic steroids, have gained attention.[2] This report describes a patient with severe asthma who became a super-responder to cycling biologic therapy using 2 biologics with mepolizumab and dupilumab.

We describe the case of a 47-year-old man who had childhood asthma. The patient had a relevant history of smoke exposure (44 pack-years) and experienced respiratory symptoms for the first time in adult age (42 years old). Nasal polyp removal surgery was performed at the age of 45 years. Although he was treated with fluticasone furoate 200 μg/vilanterol 25 μg once daily and montelukast 10 mg once daily, he continued to have recurrent asthma exacerbations, requiring the use of systemic corticosteroids. He was then referred to the Department of Airway Medicine of Mitsubishi Kyoto Hospital. He was aspirin tolerant. His body mass index was 33.0 kg/m^2. He suffered from paroxysmal nocturnal dyspnea and exacerbations of respiratory symptoms at night and in the morning. Chest radiography did not reveal lung hyperinflation. His blood eosinophil counts were 5.9% (535.7/μl), and fractional exhaled nitric oxide (FeNO) level was 46 ppb. His total immunoglobulin E (IgE) level was 127 IU/mL, and the IgE expression specific for Japanese cedar was detected. Sinus computed tomography findings showed that total Lund-Mackay score (LMS) was 14 points with predominant opacification of the ethmoid sinuses. His % predicted forced expiratory volume in one second (FEV_1) and FEV_1/forced vital capacity were 31.3% (normal value: 3.58 L) and 46.1%, respectively. We ruled out asthma-chronic obstructive pulmonary disease overlap, because his clinical symptoms were characterized by diurnal variability, the presence of a high LMS,[3] and the absence of lung hyperinflation. Fig. 1 shows the clinical course of the patient. We modified the inhaled corticosteroid therapy to four puffs of budesonide 160 μg/formoterol 4.5 μg twice daily with a technique of nasally exhaling after inhaling at "fast" inspiratory flow[4] and added tiotropium Respimat® (5 μg once daily). At 2 months after the referral, mepolizumab was started at a dose of 100 mg once monthly. However, the asthma attacks continued, requiring the use of systemic corticosteroids. Thus, administration of oral methylprednisolone 4 mg every other day was started. The asthma attacks still continued, requiring the use of additional systemic corticosteroids. At 8.5 months after the referral, omalizumab was additionally administered at a dose of 600 mg once monthly. At 3 months after the initiation of dual biologic therapy with mepolizumab and omalizumab, the oral methylprednisolone therapy was discontinued. However, the patient complained of asthma symptoms such as wheezing and coughing which were waking him up at night at a frequency of more than once a week. This required the use of systemic steroids even though total asthma control test (ACT) scores were higher than 20. This prompted a shift of medication from mepolizumab to benralizumab 30 mg every 8 weeks after three initial doses given every 4 weeks. Written informed consent was obtained from the patient for dual biologic therapy with omalizumab and mepolizumab or benralizumab and for publication of this case report. After the initiation of dual biologic therapy with omalizumab and benralizumab, no asthma exacerbations were observed. However, 8 months after the initiation of the dual biologic therapy with omalizumab and benralizumab, the blood eosinophil count increased from 0.2% to 8.4% and continued to increase. The patient experienced two asthma attacks, requiring the use of systemic corticosteroids. We shifted from dual biologic therapy with omalizumab and benralizumab to dupilumab 300 mg (initial dose of 600 mg) every 2 weeks. Thereafter, the blood eosinophil counts tended to decrease. However, 6 months after the initiation of dupilumab, the blood eosinophil counts increased again, and three asthma attacks occurred, requiring the use of systemic corticosteroid. No sensory disturbance and motor weakness were observed. Chest radiography revealed no remarkable abnormalities. At 10.5 months after the initiation of dupilumab therapy, cycling biologic therapy comprising a cycle of dupilumab administered twice every 2 weeks in a month after a single administration of mepolizumab was initiated. Total LMS was 0 point at 5 months after the initiation of cycling biological therapy. During the 12-month follow-up period, the patient did not use a short-acting beta agonist or systemic steroids, did not require emergency department visits

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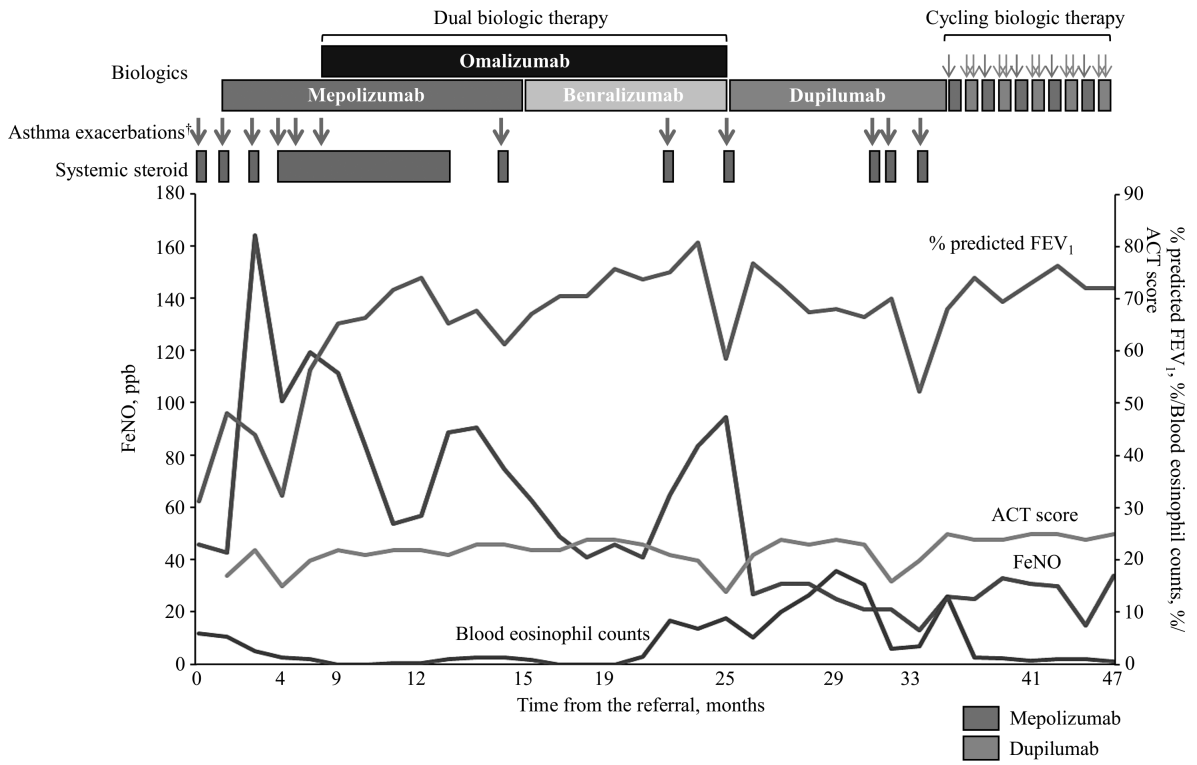

Fig. 1 Status of asthma exacerbations and the use of systemic steroids and changes in blood eosinophil counts, % predicted FEV_1, ACT score, and FeNO level after the referral. †Asthma exacerbation was defined as acute events requiring systemic steroids. ACT, asthma control test; FEV_1, forced expiratory volume in one second; FeNO, fractional exhaled nitric oxide.

or hospital admissions, and showed no decrease in ACT scores or elevation in eosinophil count. Additionally, no adverse effects occurred.

As proposed by Zervas E, et al., although omalizumab and dupilumab can be prescribed to patients with predominantly allergic asthma, mepolizumab, benralizumab, reslizumab, and dupilumab may be more suitable in those with eosinophilic asthma.[5] Thus, in asthmatic patients with both allergic and eosinophilic features in whom single biologic therapy cannot control symptoms, dual[6] or cycling[7] biologic therapy, using a combination of omalizumab or dupilumab with mepolizumab, benralizumab, reslizumab, or dupilumab can be prescribed to concomitantly control both features. Our patient presented with eosinophilic features such as elevated eosinophil counts ($>300/\mu l$), FeNO (>50 ppb), and chronic rhinosinusitis with nasal polyps, along with allergic features such as early onset, high total IgE levels (>100 IU/mL), and also tested positive for Japanese cedar-specific IgE, implying that his asthma had both allergic and eosinophilic features. Thus, dual and cycling biologic therapy, in addition to single biologic therapy, rendered the patient a "super-responder" to cycling therapy with dupilumab and mepolizumab as he did not experience loss of asthma control or any exacerbations that required systemic steroids, emergency department visits, or hospital admissions.[8]

The cost associated with cycling biologic therapy is similar to that of single biologic therapy, whereas dual biologic therapy is very expensive. On the other hand, in cycling biologic therapy, it is not known if the effects of the biologic persist even when it is not administered. Additionally, effects of the interaction of biologics are unknown in dual or cycling biologic therapy. Therefore, large randomized studies are needed to confirm the efficacy and safety of dual and cycling biologic therapy in managing severe asthma in patients who are unresponsive to single biologic therapy and to identify subgroups that are likely to benefit from these biologic therapies.

Authorship

SH, EO, and HY wrote the manuscript. SH and HY followed the patient. SH, EO, and HY read and approved the final manuscript.

Declaration of Competing Interest

Satoshi Hamada reports grants from Teijin Pharma, outside the submitted work.

Financial conflicts

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LETTER TO THE EDITOR

Intra- and interobserver agreement of inspiratory capacity during cardio pulmonary exercise test

To the Editor

The most commonly used method to measure dynamic hyperinflation (DH) is serial inspiratory capacity (IC) manoeuvres during cardiopulmonary exercise tests (CPET). The manoeuvres have to be derived from the continuously measured breathing pattern recorded. Accounting for variations in breathing pattern prior to the IC manoeuvre is crucial in order to validly calculate serial ICs upon which DH can be diagnosed.[1] This procedure of data interpretation is usually performed by a pulmonary function technician and the outcome should be independent of the individual technician.[2] The focus of this study is therefore to determine the intra- and interobserver agreement of IC measurements at rest and at peak exercise.

In a single-centre cross-sectional cohort study, 11 technicians from the Radboud University Medical Centre (Nijmegen, The Netherlands) participated. The study was conducted according to the Declaration of Helsinki and was approved by the research ethics committee of the Radboudumc (2018-4357), the study does not fall within the ambit of the Medical Research Involving Human Subjects Act.

40 CPET datasets were used from clinical practice. The technicians were all considered to be experienced assessors (+15 years) of IC measurements in the datasets (weekly assessment). To assess inter-observer agreement, 30 datasets were evaluated. The datasets were randomly chosen from a database containing all CPETs between May 2019 and December 2019. Characteristics are presented in Table 1. The other 10 datasets were revaluated at a different time to determine intra-observer agreement. In all datasets, the technicians had to determine the correct breathing level prior to a rest and peak IC manoeuvre.

The intra-class correlation coefficient (ICC) was used to determine intra- and interobserver agreement. An ICC >0.8 was considered to be close to perfect agreement. We chose a two-way random model, absolute agreement (instead of consistency), and a single measures ICC. For the intra-observer agreement we calculated the ICC per observer and determined the mean with standard deviation (SD) or median with interquartile range (IQR), dependent on normality. Normality was tested with Q-Q plots.

All the analyzed variables were normally distributed. The technicians scored a mean (SD) IC at rest of 2.33 L (0.68 L). The mean IC at peak exercise was 1.91 L (0.66 L). The ICC of the inter-observer agreement of IC rest and IC at peak exercise was 0.967 (95%CI: 0.948-0.982, p-value 0.00) and 0.976 (95%CI: 0.961-0.987, p-value 0.00), respectively.

The intra-observer agreement ranged between 0.980-1.00 for IC at rest, and 0.976-0.996 for IC at peak exercise.

In this study we determined the intra- and interobserver agreement of IC assessment by pulmonary function technicians. Guenette et al.[3] suggested that the manual calculation is subjective and could introduce an observer bias. Despites all this, we found that interpretation of IC at rest and peak exercise is excellently done by technicians. Combined with earlier studies,[4] we can now conclude that both IC measurements can be used validly. The validity of IC measurement is of great importance in the assessment of (dynamic) hyperinflation and guidance of pharmacotherapeutic and non-pharmacotherapeutic disease management. Hyperinflation is known to be stronger correlated with symptoms of patients than FEV_1 and is therefore more clinically relevant.[5, 6] To the best of our knowledge this is the first study that showed that assessment of IC is reliably done and thereby contributes to good clinical practice.

Table 1 Patient characteristics (n=30 tests), values are present as mean (SD). The non-COPD group comprises subjects referred for dyspnoe on exertion and restrictive pulmonary disease.

Age, years	62.3 (11.0)
Sex, males/females	16/14
BMI	24.9 (6.4)
FEV1, L	1.46 (0.79)
FEV1, %pred	50 (25)
FEV1/FVC%	49 (18)
COPD Gold I/II/III/IV	1/4/15/5
Non COPD	5

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Author contributions

DM and TB contributed equally to this article. DM and TB made substantial contributions acquisition and analysis of the data. BvB, DM, HvH, and TB, made substantial contributions to interpretation of the data, the conception and design, drafted or revised the article critically, provided final approval of the version to be published and agreed to be accountable for all aspects of the work.

Conflict of interest

The authors do not have a conflict of interest to declare. HvH reports an educational grant received from Chiesi Pharmaceuticals, the Netherlands.

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LETTER TO THE EDITOR

Differences in exercise capacity and health-related quality of life according to the body mass index in patients with COPD

To the Editor

Adequate pharmacological control is essential to avoid complications in patients with chronic obstructive pulmonary disease (COPD). However, it has been shown that non-pharmacological strategies such as appropriate weight and nutrition control are also important.[1]

Increased mortality has been associated in patients with COPD and low body mass index (BMI). Therefore, it is included in some multidimensional indexes.[2,3] The PLATINO study (Proyecto Latinoamericano de Investigación en Obstrucción Pulmonar), reports that low weight and BMI are independent risk factors for mortality in people with COPD; this association can be stronger when patients have greater airflow obstruction.[3]

Consequently, theories such as the "obesity paradox" have emerged, substantiating the need to know the nutritional status of the patient at the time of any intervention.[4] However, in Latin America, including Colombia, studies that relate COPD to BMI, exercise capacity and health-related quality of life (HRQoL) are scarce. Therefore, the objective of this study was to determine if there were differences in clinical variables, exercise capacity and HRQoL in patients with COPD, according to their BMI.

A descriptive cross-sectional study was carried out from July 2015 to June 2017. Patients with a previous medical diagnosis of COPD with spirometry and who entered a pulmonary rehabilitation program of the Clinica de Occidente in Cali - Colombia, were included. The Institutional Ethics Committee approved the study.

Table 1 Patients characteristics.

	Underweight=10	Normal *n* = 42	Overweight *n* = 33	Obesity *n* = 14	Total *n* = 99	*p* Value
Age (years)*	70.80 ± 7.16	71.07 ± 10.01	68.64 ± 10.11	72.43 ± 7.73	70.42 ± 9.48	0.576
Sex	8 (80.0)	27 (64.3)	23 (69.7)	9 (64.3)	67 (67.7)	0.789
Men	2 (20.0)	15 (35.7)	10 (30.3)	5 (35.7)	32 (32.3)	
Women						
Former Smoker						
Yes	9 (90.0)	35 (83.3)	28 (84.8)	12 (85.7)	84 (84.8)	0.962
No	1 (10.0)	7 (16.7)	5 (15.2)	2 (14.3)	15 (15.2)	
Home Oxygen	8 (80.0)	25 (59.5)	16 (48.5)	7 (50.0)	56 (56.6)	0.320
Yes	2 (20.0)	17 (40.5)	17 (51.5)	7 (50.0)	43 (43.4)	
No						
Socieconomic	4 (40.0)	20 (47.6)	17 (51.5)	6 (42.9)	47 (47.5)	0.958
Status	5 (50.0)	19 (45.2)	12 (36.4)	7 (50.0)	43 (43.4)	
Low	1 (10.0)	3 (7.1)	4 (12.1)	1 (7.1)	9 (9.1)	
Medium						
High						
Exacerbations						
Yes	7 (70.0)	23 (54.8)	16 (48.5)	6 (42.9)	52 (52.5)	0.562
No	3 (30.0)	19 (45.2)	17 (51.5)	8 (57.1)	47 (47.5)	

*Mean ± Standard Deviation

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Table 2 Clinical characteristics

	Underweight *n* = 10	Normal *n* = 42	Overweight *n* = 33	Obesity *n* = 14	Total *n* = 99	*p* Value
HEIGHT (meters)	1.61 ± 0.1	1.62 ± 0.1	1.62 ± 0.1	1.63 ± 0.1	1.62 ± 0.1	0.938
WEIGHT (kg)	46.9 ± 6.8	57.7 ± 8.8	71.1 ± 6.6	86.7 ± 7.6	65.2 ± 13.9	0.000
mMRC	3.6 ± 0.5	2.8 ± 1.1	3.0 ± 1.1	2.8 ± 1.2	2.9 ± 1.1	0.186
FEV1 (% PREDICTED)	40.5 ± 20.6	43.6 ± 14.2	42.4 ± 16.2	43.8 ± 13.4	42.9 ± 15.3	0.938
FVC (% PREDICTED)	66.4 ± 26.1	68.4 ± 19.5	66.4 ± 24.0	70.2 ± 15.5	67.8 ± 21.0	0.940
FEV1/FVC	57.8 ± 14.3	61.6 ± 9.4	62.3 ± 7.5	60.6 ± 9.9	61.3 ± 9.4	0.613
6MWT DISTANCE (meters)*	225.2 ± 82.9	304.9 ± 106.7	336.9 ± 107.1	292.7 ± 116.8	305.8 ± 109.4	0.038
HR BASAL (bpm)	85 ± 12.6	84.2 ± 14.8	82.2 ± 11.9	78.3 ± 7.5	82.8 ± 12.8	0.456
HR FINAL (bpm)	104.3 ± 13.2	108.1 ± 16.4	108.7 ± 14.7	103.6 ± 16.2	107.3 ± 15.4	0.681
RR BASAL (bpm)	21 ± 2.2	22.3 ± 12.9	19.3 ± 4.6	19.1 ± 2.7	20.7 ± 8.9	0.469
RR FINAL (bpm)	26.1 ± 4.7	25.5 ± 5.2	26.3 ± 4.5	26.2 ± 3.1	25.9 ± 4.6	0.889
BORG	0.5 ± 0.9	0.4 ± 0.7	0.5 ± 0.9	0.3 ± 0.6	0.4 ± 0.8	0.901
BORG FINAL	3.1 ± 2.2	2.2 ± 1.9	2 ± 1.8	1.7 ± 1.7	2.2 ± 1.9	0.306
SpO2 BASAL (%)	93 ± 2.6	94.1 ± 2.8	94.1 ± 3.4	93.9 ± 2	93.9 ± 2.9	0.728
SpO2 FINAL (%)	87.2 ± 4.1	87.9 ± 5.9	87.7 ± 6.5	89 ± 6.6	87.9 ± 6.0	0.891
DESATURATION (%)	5.8 ± 3.5	6.3 ± 5.1	6.4 ± 4.6	4.9 ± 5.2	6.1 ± 4.8	0.784
VO2e (ml/kg/min)**	6.3 ± 2.6	8.4 ± 1.9	8.5 ± 2.3	7.7 ± 2.2	8.1 ± 2.3	0.032
HAD ANSIETY	7.9 ± 4.5	6 ± 4.5	6.70 ± 4.9	6.1 ± 5.4	6.4 ± 4.7	0.685
HAD DEPRESSION	6.1 ± 4	5.4 ± 3.8	5.9 ± 4.9	3.9 ± 3	5.4 ± 4.2	0.484
SGRQ SYMPTOMS	50.4 ± 23.1	47.5 ± 17.7	50 ± 20.9	49.6 ± 23.3	48.9 ± 19.9	0.947
SGRQ ACTIVITY***	75.2 ± 19.3	59.6 ± 23.5	62.7 ± 21.8	48.6 ± 24.3	60.7 ± 23.3	0.043
SGRQ IMPACT	47.3 ± 21.9	37.5 ± 14.9	43.9 ± 19.3	38.9 ± 20	40.8 ± 18	0.279
SGRQ TOTAL	57.6 ± 18.1	47.5 ± 14	52.5 ± 18	46.7 ± 18.4	50.1 ± 16.6	0.226

mMRC: modified Medical Research Council, FEV1: Forced Expired Volume in the First Second, FVC: Forced Vital Capacity, FEV1/FVC: Forced Expired Volume in the First Second/Forced Vital Capacity ratios, SpO2: Oxygen Saturation, VO2e: Consumption Oxygen estimated, 6MWT Distance: 6-Minutes Walk Test Distance, HAD: Hospital Anxiety and Depression Scale, SGRQ: Saint George Respiratory Questionnaire, HR: Heart Rate, RR: Respiratory Rate

* Statistically significant intergroup differences Underweight vs Normal *p*-value = 0.010 and Underweight vs Overweight *p*-value = 0.015

** Statistically significant intergroup differences Underweight vs Overweight *p*-value = 0.045

*** Statistically significant intergroup differences Underweight vs Obesity *p*-value = 0.038

The patients were divided into four groups, according to the BMI Classification of the World Health Organization (WHO): Underweight (BMI less than 18.5 kg/m^2), Normal weight (BMI $\geq$ 18.5 and less than 25.0 kg/m^2), Overweight (BMI $\geq$ 25.0 and less than 30.0 kg/m^2), and Obesity (BMI $\geq$30.0 kg/m^2).

Spirometry and baseline dyspnea with the modified medical research council (mMRC) scale were assessed. At the beginning and at the end of the 6-minute walk test (6MWT), patients were measured for distance, VO2 estimated (3.5 ml/kg/min + (speed m/min $\times$ 0.1), heart rate, respiratory rate, modified Borg dyspnea scale, and oxygen saturation (SpO2).

Anxiety/Depression was evaluated using the Hospital Anxiety and Depression Scale (HADS) questionnaire (8 as the cut-off point). Health-Related Quality of Life (HRQoL) was measured using the St. George's Respiratory Questionnaire (SGRQ).

The descriptive analysis was carried out for all the variables. To determine the differences according to the BMI group, ANOVA tests were performed, and Dunnett's T3 test confirmed the inter and intragroup differences. As a result, 99 patients organized according to BMI were linked into Underweight *n* = 10, Normal *n* = 42, Overweight *n* = 33, Obesity *n* = 14.

Table 1 shows the patients' characteristics. Table 2 depicts the BMI and clinical variables. The variables distance in 6MWT, VO2e, and the Activity domain of the SGRQ showed statistically significant differences within the groups. Regarding the distance in the 6MWT, the BMI of the underweight and obese groups showed intergroup differences (95% CI under-Normoweight (7.10—152.38) under-

Overweight (37.11−186.19), compared with Normal and overweight BMI. There was a similar finding for VO2e. The Activity domain of the SGRQ showed statistically significant differences.

These findings reveal that patients who were overweight, according to the BMI, had better results in terms of exercise capacity and HRQoL. These changes could have several explanations: patients with sustained hypoxia and oxidative metabolism could experience muscle wasting.[5] Our study supports this thesis and identifies the need to strengthen patient care strategies, supporting the use of drug treatment accompanied with non-drug strategies such as pulmonary rehabilitation and nutritional intervention (e.g., proper food and supplementation).

Regarding the evaluation of functional exercise capacity, it was found that the overweight group reached more distance on the 6MWT and obtained greater VO2e, followed by the normal BMI group. Physical inactivity is linked to early loss and wasting of muscle function, which can further decrease physical activity and exercise tolerance.[6] This could also explain why patients deteriorated more significantly in the Activity domain of the HRQoL.[7] In addition, the literature reports that obesity in this type of patient allows them to have greater metabolic reserve, which leads to better survival rates and fewer exacerbations and emergency visits.[8]

According to the results, patients with a diagnosis of COPD who were overweight had better exercise capacity and HRQoL in the activities domain compared to patients with normal weight and malnutrition. However, these findings will have to be confirmed with large intervention studies.

Conflicts of interest

The authors have no conflicts of interest

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LETTER TO THE EDITOR

Pulmonary function tests: The patient's perspective

To the Editor

The SARS-COV2 virus emerged in December 2019, in China.[1] It quickly crossed borders and spread worldwide, affecting a large number of individuals. It was declared a pandemic on March 11, 2020.[2] It is a highly contagious virus, transmitted person-to-person, not only by inhaling droplets and aerosols, but also by direct contact through surfaces.[3]

Pulmonary function tests (PFT), especially forced manoeuvres and possible cough induction, are aerosol generating procedures[4,5] that can spread viral particles, thus presenting a high risk of contamination and cross-infection between patients and healthcare workers.[6]

Several scientific societies developed rules and recommendations on the reorganization and conditions of respiratory function laboratories with regard to the use of personal protective equipment by the technicians who carry out the PFTs, the ventilation of the rooms, the use of disposable materials, the cleaning of equipment and surfaces, the distance between tests, the absence of companions, the use of surgical masks only removed for the exam and the use of antimicrobial filters.[7-12]These procedures aim to minimize the risk of infection by direct and indirect contamination of patients, workers, and environment, maximizing everyone's safety.

Despite these measures, we believe that patients' perception and experience could differ from those of the healthcare workers. Thus, the aim of our study was to identify the fears and constraints experienced by patients, during the PFTs.

This study was carried out in April 2021 at the Respiratory Function Laboratory of the Centro Hospitalar de Vila Nova de Gaia/Espinho. It was a cross-sectional study, and the patients were selected consecutively after undergoing the procedure (PFT). All the invited individuals agreed to participate in the study.

The collected data was analysed using the SPSS Statistics program, using the Student T, Chi-squared, and Fisher's exact tests, with a p-value of <0.05 considered statistically significant.

The sample of this study is composed by 103 patients whose general characteristics are shown in Table 1.

It was a heterogeneous sample, mainly composed of males (59%) and people whose level of education was only the first cycle of basic education (45%). Also, the majority had previously undergone a PFT (84%), had not been infected by SARS-COV2 (89%), and had not yet been vaccinated (66%).

Regarding the main objective of the study, we observed that 90% ($N = 93$) of the patients denied fear of undergoing a PFT at admission, and after the procedure the level of fear of a significant part of them (64%) remained the same, while 33% reported a decrease in their fear, having expressed a feeling of confidence and security with the measures implemented in the laboratory. Only one patient considered missing the exams, despite the fact that we are still in a period of pandemic.

As for the group of individuals who said they were afraid of undergoing a PFT ($N = 10$) at admission, the mean age was 53.8 years, and there was no gender predominance. Among these individuals, the majority had only completed the first

Table 1 Patients' characteristics.

	Total ($N = 103$)	
Age	60.6 (15.88)	
Gender	Male 59%	($N = 61$)
	Female 41%	($N = 42$)
Level of education	1st cycle 45%	($N = 46$)
	2nd cycle 20%	($N = 21$)
	3rd cycle 11%	($N = 11$)
	Secondary Education 11%	($N = 11$)
	Higher Education 13%	($N = 14$)
First time PFT	Yes 16%	($N = 16$)
	No 84%	($N = 87$)
Previous SARS-COV2 infection	Yes 11%	($N = 11$)
	No 89%	($N = 92$)
SARS-COV2 vaccine	Yes 34%	($N = 35$)
	No 66%	($N = 68$)
Fear to perform PFT	Yes 10%	($N = 10$)
	No 90%	($N = 93$)

Age: mean (standard deviation); Other variables presented in relative and absolute frequency. PFT: Pulmonary function tests.

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Table 2 Comparison between patients with and without fear of undergoing pulmonary function tests.

	Patients Without Fear (*N* = 93)		Patients With Fear (*N* = 10)		P *value*
Age	61.3 (15.45)		53.8 (19)		0.155
Gender	Male 60%	(*N* = 56)	Male 50%	(*N* = 5)	0.532
	Female 40%	(*N* = 37)	Female 50%	(*N* = 5)	
Level of education	1st cycle 45%	(*N* = 42)	1st cycle 50%	(*N* = 5)	0.616
	2nd cycle 18%	(*N* = 18)	2nd cycle 20%	(*N* = 2)	
	3rd cycle 11%	(*N* = 9)	3rd cycle 20%	(*N* = 2)	
	Secondary Ed 12%	(*N* = 11)	Secondary Ed 0%	(*N* = 0)	
	Higher Ed 14%	(*N* = 13)	Higher Ed 10%	(*N* = 1)	
First time PFT	Yes 18%	(*N* = 18)	Yes 0%	(*N* = 0)	0.203
	No 82%	(*N* = 75)	No 100%	(*N* = 10)	
Previous SARS-COV2 infection	Yes 12%	(*N* = 11)	Yes 10%	(*N* = 1)	1
	No 88%	(*N* = 82)	No 90%	(*N* = 9)	
SARS-COV2 vaccine	Yes 34%	(*N* = 32)	Yes 30%	(*N* = 3)	1
	No 66%	(*N* = 61)	No 70%	(*N* = 7)	
Afraid after PFT	Less/ Equal 96.8%	(*N* = 90)	Less/ Equal 100%	(*N* = 10)	1
	More 3.2%	(*N* = 3)	More 0%	(*N* = 0)	

Age: mean (standard deviation); other variables presented in relative and absolute frequency. Ed: education; PFT: Pulmonary function tests.

cycle of education (50%) and 50% had a diagnosis of asthma. There was one case of previous infection by SARS-COV2, and 30% had already been vaccinated. The most frequently mentioned fear factors were the need to remove the mask, to put their mouth in the equipment, being in a closed space, and the presence of other patients in the waiting room. After undergoing a PFT, the level of fear for half of these patients remained the same, but the rest reported a decrease.

The comparative analysis between the two groups of patients — those who admitted and those who denied fear of undergoing a PFT at admission — did not show significant differences in relation to the variables assessed and no association was observed between any of the characteristics studied and the fear of undergoing a PFT, as noted in Table 2.

On the other hand, there were 3 patients who were not afraid of undergoing a PFT at admission who showed an increase in fear after undergoing the tests. These individuals had similar characteristics, such as young age (mean age of 32 years), a diagnosis of asthma, secondary education, and, in two cases, it was their first PFT. Despite all the procedures, they revealed discomfort during the experience, especially due to the need to remove the mask, put their mouths in the equipment, being in a closed space, mentioning as well that the waiting room was too small and did not allow for the recommended social distancing.

Despite strategies used to identify individuals suspected of being infected with SARS-COV2, there might be people with subclinical disease who can transmit it.[11] Previous studies have shown there are differences between men and women regarding the risk of contracting SARS-COV2 infection, severe complications after infection, death, and psychological and emotional impact related to biological, social, economic, work, behavioural, and lifestyle factors.[13] In our study, the analysed variables did not show a correlation with the fear felt by patients before and after undergoing the tests. Thus, we conclude that undergoing a PFT, in this period, constituted an individual and unique experience for each patient that results from their pathological background, but will be influenced by their beliefs, values, along with their social, economic, and cultural environment, reflecting each person's subjectivity in the way they face this pandemic.

A limitation of the study was the fact that 9.3% of patients missed the PFTs during the analysed period, and although we do not know the reasons for this — which might be related to the fear of contamination by SARS-COV2 — we found that these absences were consistent with those during the pre-pandemic period.

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LETTER TO THE EDITOR

ROS-1 TKI for the treatment of concurrent sarcomatoid transformation and acquired ROS-1 F2004C mutation in a lung adenocarcinoma patient

To the Editor

ROS1 gene rearrangement is one of the druggable driver oncogenes that accounts for 1—2% of all non-small cell lung cancer (NSCLC), almost exclusively adenocarcinoma.[1] Tumors harboring ROS1 gene rearrangements respond well to ROS1 tyrosine kinase inhibitors (TKIs), including crizotinib, ceritinib, entrectinib, and lorlatinib; however, resistance inevitably develops. Possible resistance mechanisms include genetic alteration of the drug target, activation of bypass signaling, or histological transformation to sarcomatoid carcinoma or small cell carcinoma.[2] Here, we report a case of lung adenocarcinoma with CD74-ROS1 gene rearrangement that underwent sarcomatoid transformation during disease progression after crizotinib treatment. A tumor genome study also revealed a ROS1 F2004C mutation, and the patient was successfully treated with lorlatinib.

A 43-year-old non-smoker female was diagnosed with right upper lung adenocarcinoma (Fig. 1A) with an initial presentation of chronic cough for 3 months. The initial staging was cT4N3M1c with malignant pericardial effusion, spine metastasis, and solitary brain metastasis. ROS1 rearrangement was confirmed by both immunohistochemical stains (Fig. 1A) and fluorescence in situ hybridization (FISH). The patient was treated with cisplatin and pemetrexed for 1 cycle and then shifted to crizotinib due to the rapid progression of malignant pericardial and pleural effusion. The patient was kept on crizotinib for 10 months until an asymptomatic left-sided pleural mass was found during regular chest CT follow-up. A percutaneous sono-guided biopsy was performed at the pleural mass. Pathology showed pleomorphic spindle-shaped cells in solid sheets (Fig. 1C), with diffuse immunoreactivity for both cytokeratin (CK) and ROS1 (Fig. 1D).

We speculated that the failure of first line chemotherapy treatment may be attributable to a larger tumor burden at the time; since chemotherapy often takes several weeks to achieve a clinical response, the patient received cisplatin and pemetrexed again. However, 3 weeks later, the patient was admitted to our emergency room (ER) due to severe dyspnea. Cardiac tamponade was diagnosed, and sudden collapse occurred with pulseless electrical activity noted during pericardiocentesis. The vital signs and consciousness gradually recovered after pericardiocentesis and intubation. Chest CT showed rapid progression of multiple pleural masses, malignant pericardial and pleural effusion, and lymphangitic carcinomatosis (Fig. 2B). After discussion with her family, lorlatinib 100mg was given via nasogastric tube. A pleural tumor specimen obtained previously was sent for next-generation sequencing (NGS) (Oncomine Focus Assay platform), and the result revealed a CD74-ROS1 fusion and ROS1 F2004C mutation. Extubation was performed 8 days after lorlatinib administration, and serial CXR (Fig. 2A) showed tumor regression, further confirmed by chest CT (Fig. 2B). The disease remained under control for 6 months.

Pulmonary sarcomatoid carcinoma is a rare histology type, accounting for only 0.1—0.4% of NSCLC.[3] Lung adenocarcinoma with sarcomatoid transformation is even rarer. The optimal treatment modality for these patients is undecided. In a study from the Mayo Clinic involving the largest cohort of 127 patients with pulmonary sarcomatoid carcinoma, the response rate to palliative chemotherapy was only 8%, and the median overall survival was 7.7 months in stage IV disease.[4] Recent studies have shown that pulmonary sarcomatoid carcinoma may harbor druggable oncogenes, such as MET-14 skipping, EGFR mutation, ALK translocation, and BRAF and ROS1

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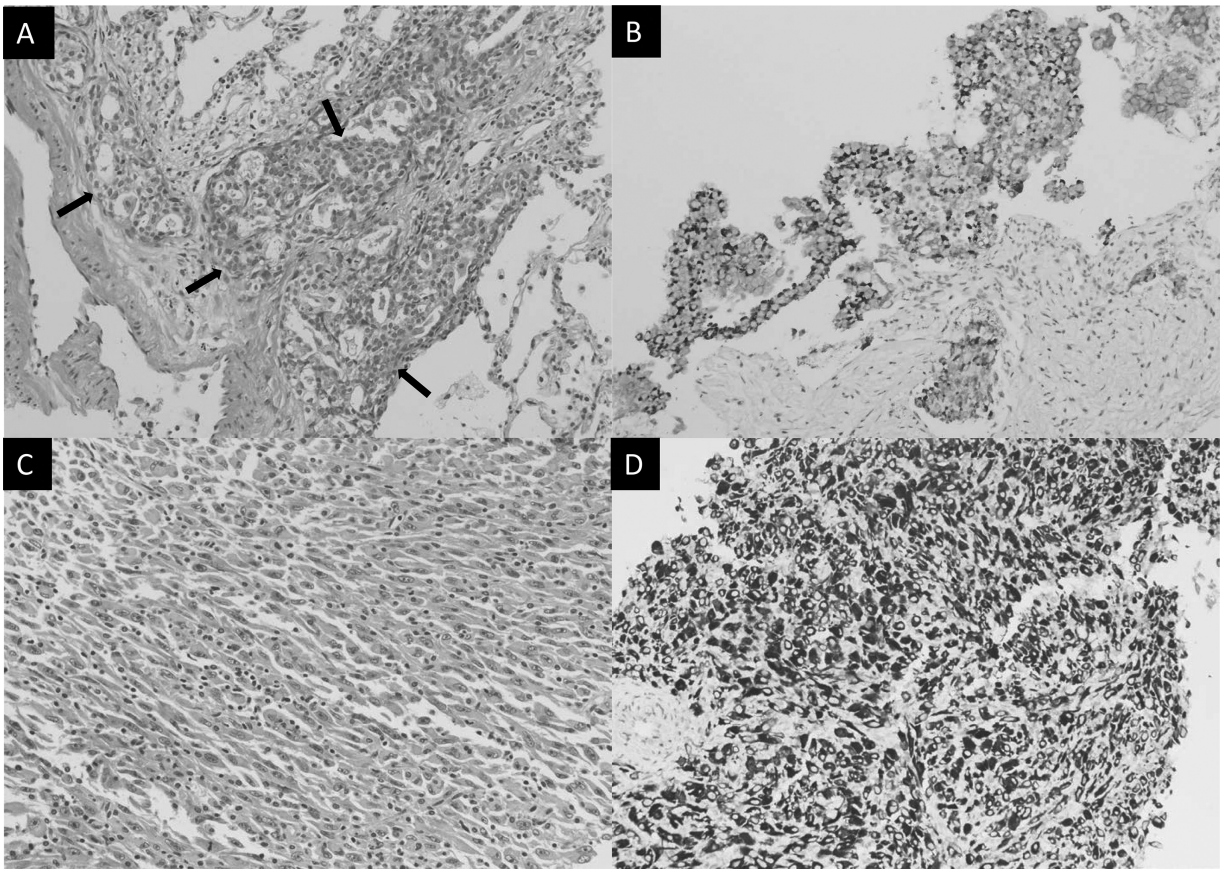

Figure 1 Pathology of the initial right upper lung tumor (A, B) and left pleural mass upon progression (C, D). (A) Hematoxylin and eosin stain showed round tumor cells (arrows) forming a glandular structure and solid nest. (B) Positive staining in ROS1 immunostaining. (C) Hematoxylin and eosin staining showed pleomorphic spindle-shaped tumor cells. (D) Tumor cells show positive staining in ROS1 immunostaining.

rearrangement.[5] Several case reports have shown that such tumors may respond to relevant TKIs.

Crizotinib resistance mechanism is widely studied both in ALK and ROS1 rearrangement, including "on-target" crizotinib binding site secondary gene mutation, with ROS1 G2032R being most frequently identified. ROS1 F2004C/V was another acquired on-target mutation postulated to be treated by lorlatinib, another potent ALK/ROS1 TKI, using an in vitro model.[6] Sarcomatoid transformation is another potential crizotinib resistance mechanism. Kobayashi et al. reported a case of ALK translocation adenocarcinoma in which disease progression occurred during crizotinib treatment following a biopsy that confirmed sarcomatoid transformation.[7]

In conclusion, Lorlatinib overcame both histological transformation and crizotinib binding site secondary mutation, suggesting that these sarcomatoid transformed cancer cells are still "addicted" to the ROS1 pathway. These findings highlight that histological transformation and other acquired mutations may coexist during disease progression; hence, comprehensive genetic testing is clinically valuable in determining resistance mechanisms to decide the next-line treatment.

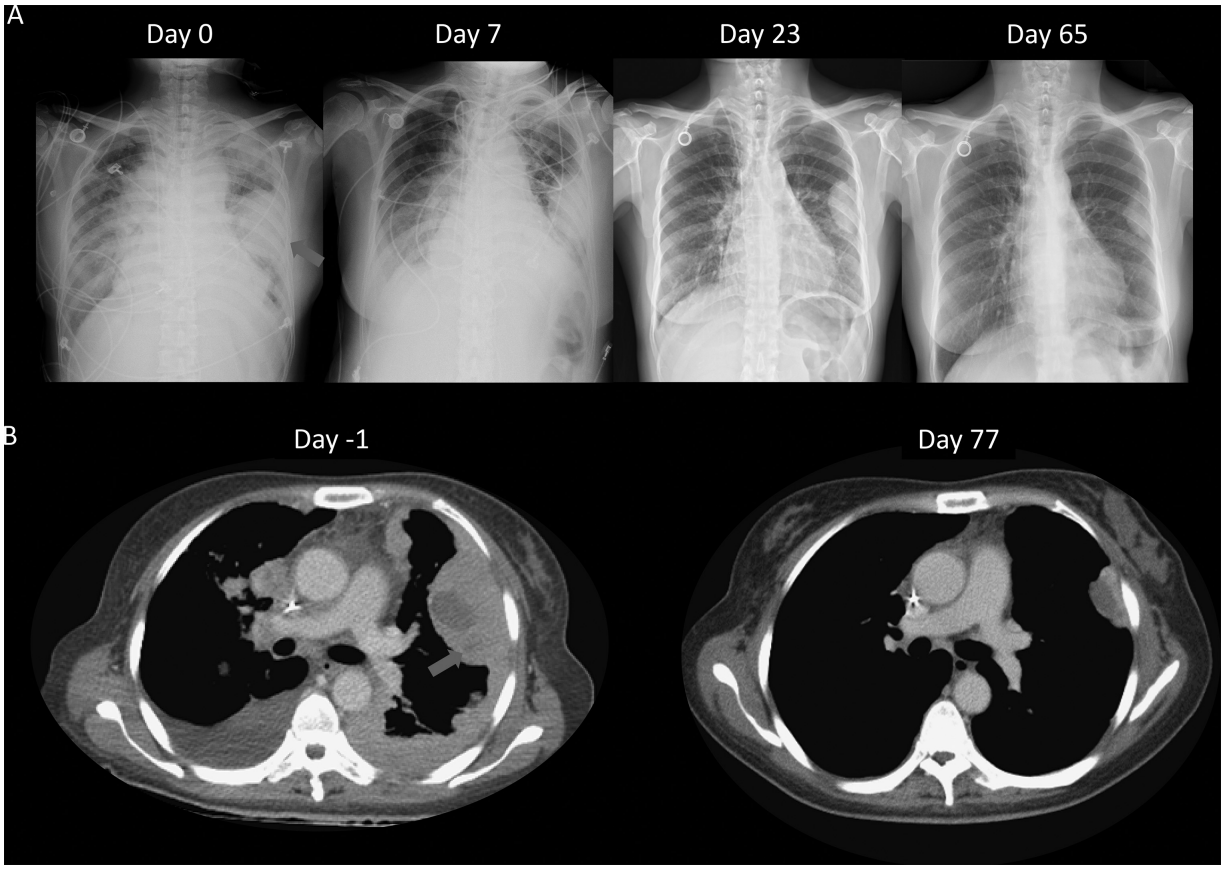

Figure 2 (A) Serial chest X-ray images from the initiation of lorlatinib treatment (day 0) to 65 days after initiation (day 65). Arrow: pleura mass underwent biopsy. (B) CT before and after lorlatinib treatment.

Conflicts of interest

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LETTER TO THE EDITOR

Lung cancer in young patients: natural history, biology and prognosis

To the Editor

Lung cancer (LC) is the leading cause of cancer mortality in Portugal and although more frequent in male and older patients (pts), 0.6—13% of LC diagnosis occur in young pts.[1] The definition of young patient is not clear, varying between less than 35 years to less than 50 years.[1,2] Several studies have been published regarding LC in young pts, suggesting an increased percentage of female[1—4] and non-smokers pts,[1,5] a longer duration of symptoms,[5] a higher frequency of adenocarcinoma[1—5] rather than squamous cell carcinoma and a higher frequency of advanced disease at diagnosis.[2,4,6] It is still controversial whether younger pts have similar,[6] better[1—4] or worse[5] outcomes than older pts and recent studies have suggested that young pts with non-small cell lung carcinoma (NSCLC) harbor more driver mutations than older pts.[7]

In order to understand whether or not LC in younger pts is a genetically unique disease with a particular natural history, biology and prognosis, we retrospectively performed a comprehensive and comparative analysis of younger versus older LC pts diagnosed in our institution from January 2014 to April 2020. Patients were included in the young cohort if their age at diagnosis was greater than two standard deviations less than median age at diagnosis,[2] which in our study meant pts aged 42 years or younger. Patients´ clinical and pathological features and clinical outcomes were evaluated. Categorical characteristics were compared using the Chi-square test and continuous variables were compared using the Kaplan- Meier method. A p value less than 0.05 was considered significant.

We identified 1315 pts with LC: 43 (3.3%) pts were included in the young cohort (median age at diagnosis was 37.9 years, 29—42) and 1272 (96.7%) pts were included in the old cohort (median age at diagnosis was 65.8 years, 43—95). Younger pts were more likely to be female, have an Eastern Cooperative Oncology Group performance status of 0—1, have fewer comorbidities at diagnosis and to be never-smokers, than older patients. Similar rates of symptomatic disease, node positive disease and metastatic disease were seen in both cohorts (Table 1).

Lung cancer in the young versus old cohorts was equally likely to be adenocarcinoma, more likely to be carcinoid tumor and less likely to be squamous cell carcinoma. The frequency of *EGFR* and *ALK* variants was similar in both cohorts. Median follow-up time was longer in the young cohort (16.6 months versus 13.4 months, $p = 0.123$). There were 46.5% versus 53.8% deaths registered in the young and old cohorts, respectively. Median overall survival (OS) was better in the young cohort, but the difference was not significant (9.2 months versus 8.4 months; $p = 0.166$). No deaths were documented in clinical stages I and II in the young cohort. Median OS was better in younger pts with clinical stages I, II and IV LC and worse in younger pts with clinical stage III LC, but differences were not significant ($p = 0.245$; $p = 0.332$; $p = 0.088$; $p = 0.459$, respectively).

Our findings are consistent with previous studies suggesting that younger pts with LC are more likely to be female,[1—4] fitter,[2] healthier[1,2] and never-smokers.[1—5] Adenocarcinoma was the most common histopathology in both age groups but unlike other reports,[1—5] an increased likelihood of adenocarcinoma in younger pts was not found. We also report a lower incidence of driver mutations in comparison to previous studies[7] and an increased rate of *EGFR* and *ALK* variants in younger pts was not found. Moreover, we reported a non-significant improved outcome in younger pts and median OS in both younger and older pts was inferior in comparison to previous studies. Our study was not, however, restricted to NSCLC as most previous studies were and that may explain the differences found. The small number of younger pts may

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Table 1 Clinical and pathological features of LC pts in the young versus old cohorts.

Clinical and pathological features		Young cohort n(%)	Old cohort n(%)	*p value (Chi square)*
Sex	Male	22 (51.2)	854 (67.1)	0.028903
	Female	21 (48.8)	418 (32.9)	
ECOG PS	0-1	40 (93)	996 (78.3)	0.02022
	2	1 (2.3)	153 (12)	(ECOG PS 0−1 vs 2−4)
	3-4	2 (4.7)	123 (9.7)	
Smoking history	Ever-smoker	28 (65.1)	1033 (81.2)	0.008556
	Never-smoker	15 (34.9)	239 (18.8)	
Comorbidities at diagnosis	Yes	6 (13.9)	875 (68.8)	<0.00001
	No	37 (86.1)	397 (31.2)	
Symptoms at diagnosis	Yes	34 (79.1)	888 (69.8)	0.192091
	No	9 (20.9)	384 (30.2)	
Node disease at diagnosis	Node positive	31 (72.1)	961 (75.5)	0.897711
	Node negative	12 (27.9)	311 (24.5)	
Metastatic disease at diagnosis	Yes	26 (60.5)	651 (51.2)	0.230808
	No	17 (39.5)	621 (48.8)	
	NSCLC	32 (74.4)	1078 (84.7)	
	Adenocarcinoma	28 (65.1)	750 (58.9)	0.4194 for adenocarcinoma
	Squamous cell	2 (3.8)	245 (19.3)	
	Large cell	0	6 (0.5)	
	Adenosquamous	0	12 (0.9)	0.00001 for carcinoid tumor
	Sarcomatous	1 (2.3)	5 (0.4)	
Histologic diagnosis	NOS	1 (2.3)	60 (4.7)	
	Neuroendocrine	11 (25.6)	165 (13)	0.015847 for squamous cell
	Small cell	4 (9.3)	122 (9.6)	carcinoma
	Large cell	1 (2.3)	28 (2.2)	
	Carcinoid	6 (14)	15 (1.2)	
	Other		29 (2.3)	
	EGFR	2 (6.7)	160 (17.5)	0.1213 for EGFR mutations 0.1987
	ALK	3 (10)	44 (4.8)	for ALK mutations
Driver mutations	*ROS1*	1 (3.3)	4 (0.4)	
	BRAF	1 (3.3)	3 (0.3)	

also explain survival differences. Prospective multicentre studies are needed.

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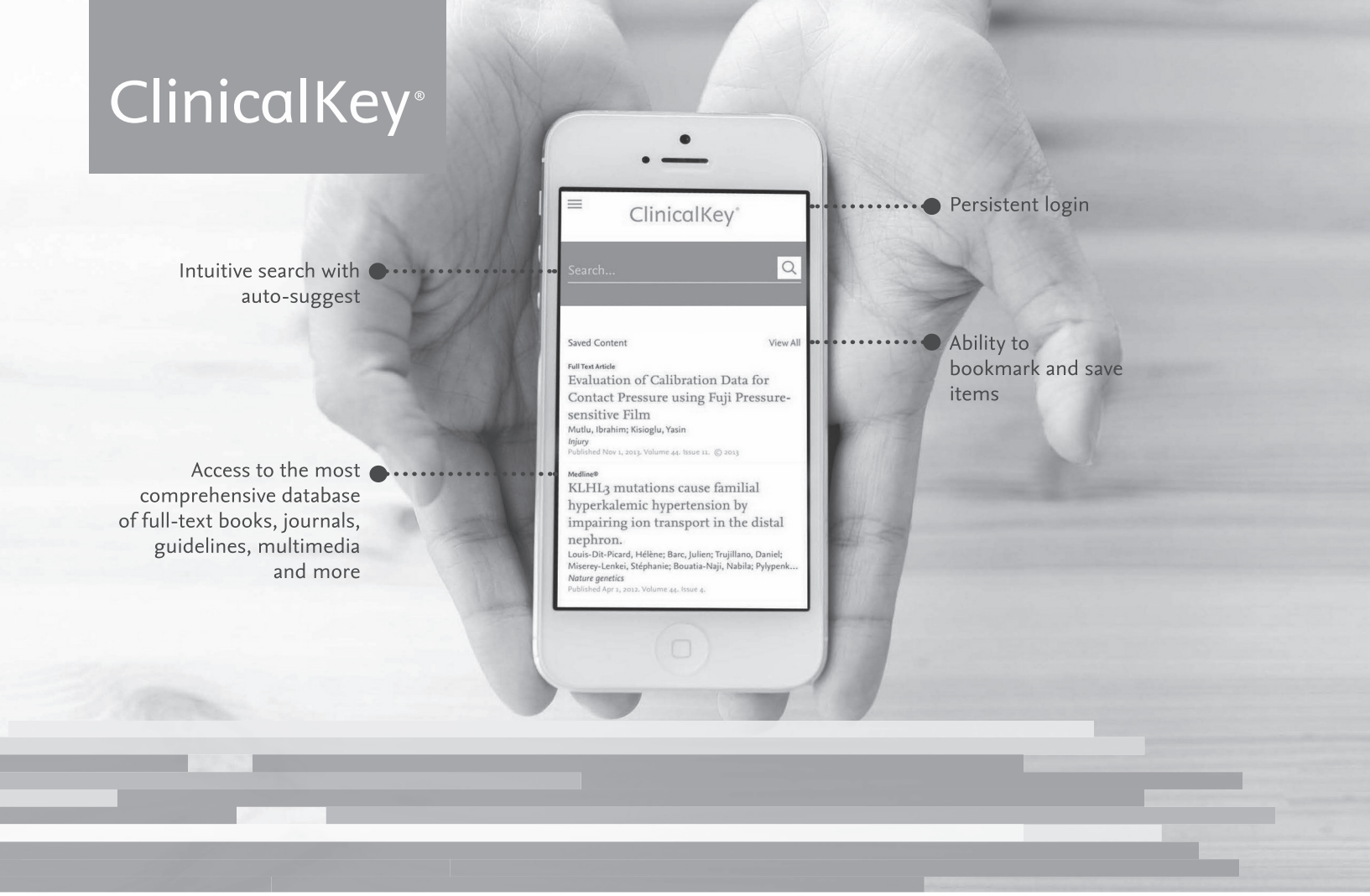

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