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Utility of Individualised FeNO in Diagnosing Asthma in Primary Care Settings: A Secondary Analysis of EPI-ASTHMA

Diana Isabel Sanches Gomes

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I want to think that one day it will all fade away, that it will just be a small scar, that I will look at it and it will teach me something. As my sister Helena's favourite writer, Fernando Pessoa says, “Eu tenho em mim todos os sonhos do mundo”, and on that day when I allow myself to dream, I know that I have allowed myself to be human.

Abstract

Introduction and objectives

Fractional exhaled nitric oxide (FeNO) has emerged as a non-invasive biomarker for assessing eosinophilic airway inflammation, offering potential support for asthma diagnosis at primary care. However, standard diagnostic cut-offs fail to account for significant inter-individual variation. To overcome this limitation, this study explored the diagnostic utility of individualised FeNO values, derived from previously developed reference equations and compared to fixed cut-offs, for asthma diagnosis in primary care settings.

Materials and Methods

This is a secondary analysis of the EPI-ASTHMA study conducted in 38 Portuguese primary care centres, which included adults participating in both stage 1 and stage 2 of the study. Clinical assessments included spirometry, FeNO measurements, and blood eosinophil counts. Individualised FeNO values were calculated using the Lambda-Mu-Sigma method, adjusted for age, sex and height. Diagnostic accuracy of individualised cut-offs was assessed using area under the Receiver Operating Characteristic curve (AUC) and compared against the American Thoracic Society (ATS) recommended absolute cut-offs (>25ppb, >50 ppb).

Results

A total of 1,383 participants (median[p25-p75]: 54 [43-65] years) were analysed, of whom 60.2% were female, and 11.7% were current smokers. Asthma was diagnosed in 258 (18.7%) participants, who had significantly higher FeNO levels compared to the non-asthma group (23.0 [13.0-43.75] ppb vs 16.0 [11.0-25.0] ppb, p<0.001). The diagnostic utility of FeNO was moderate for both absolute (AUC 0.63; 95% confidence interval (CI) 0.58-0.67) and individualised-predicted (AUC 0.64; 95%CI 0.60-0.69) values. The 202% predicted individualised cut-off demonstrated the highest specificity (84.2%), while the 100% predicted individualised cut-off yielded the best sensitivity (68.4%) and negative predictive value (86.8%). Thus, 100% and 202% individualised

predicted values can be used to rule-out asthma. The 50 ppb cut-off retained the highest specificity (94.3%), although with low sensitivity (22.5%).

Conclusion

FeNO demonstrates moderate performance as a stand-alone tool for asthma diagnosis. Individualised FeNO cut-offs improve specificity and may better support clinical decision-making in ruling out asthma in primary care centres and potentially lead to more accurate diagnoses and improved patient management.

Key words: exhaled nitric oxide, biomarkers, airway inflammation, asthma

Resumo

Introdução e objetivos

A fração de óxido nítrico exalado (FeNO) emergiu como um biomarcador não invasivo para avaliar a inflamação eosinofílica das vias aéreas, oferecendo potencial apoio ao diagnóstico de asma nos cuidados de saúde primários. No entanto, os valores de corte de diagnóstico padronizados não consideram a significativa variação interindividual. Para superar esta limitação, este estudo explorou a utilidade diagnóstica dos valores individualizados do FeNO, derivados de equações de referência previamente desenvolvidas (p. ex.: o método Lambda-Um-Sigma), e comparou-os com os valores de corte fixos para o diagnóstico de asma.

Material e Métodos

Esta é uma análise secundária do estudo EPI-ASTHMA realizado em 38 centros de cuidados de saúde primários em Portugal, que incluiu adultos participantes nas fases 1 e 2. As avaliações clínicas compreenderam espirometria, medições do FeNO e contagem de eosinófilos no sangue. Os valores individualizados de FeNO foram calculados utilizando o método Lambda-Um-Sigma já existente, ajustados para a idade, sexo e altura. A precisão diagnóstica dos valores de corte individualizados foi avaliada utilizando curvas ROC (receiver operating characteristic) e comparada com os valores de corte absolutos recomendados pela American Thoracic society (ATS). Propusemos dois valores para a percentagem prevista (% pred) para caraterizar os valores individualizados de FeNO, nomeadamente, 100% pred e o 202% pred, para excluir o diagnóstico de asma.

Resultados

Foi incluído um total de 1383 participantes (mediana [p25-p75] 54 [43-65] anos), dos quais 60,2% eram do sexo feminino e 11,7% eram fumadores ativos. A asma foi diagnosticada em 258 (18,7%) participantes, que apresentavam níveis de FeNO significativamente mais elevados em comparação com o grupo de participantes sem asma (23,0 [13,0-43,75] ppb vs 16,0 [11,0-25,0] ppb, p<0,001). A utilidade diagnóstica

do FeNO foi moderada tanto para os valores previstos (AUC 0,64 [IC95% 0,60-0,69]) quanto para os valores absolutos (AUC 0,63 [IC95% 0,58-0,67]). O cut-off individualizado previsto a 202% demonstrou a maior especificidade (84,2%), enquanto que o cut-off individualizado previsto a 100% produziu a melhor sensibilidade (68,4%) e o valor preditivo negativo (86,8%). O cut-off absoluto ATS 50 ppb manteve a especificidade mais elevada (94,3%), embora com baixa sensibilidade (22,5%).

Conclusão

O FeNO demonstra um desempenho diagnóstico moderado como instrumento autónomo para a asma. Os pontos de corte individualizados do FeNO melhoram a especificidade e podem apoiar melhor o processo de tomada de decisão clínica na exclusão de asma, nos centros de cuidados de saúde primários e, potencialmente, conduzir a terapêuticas e diagnósticos mais exatos.

Palavras-chave: óxido nítrico, biomarcadores, inflamação, asma

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Abbreviations

ATS: American Thoracic Society

AUC: Area Under the Curve

BEos: Blood Eosinophils Count

BMI: Body Mass Index

CI: Confidence Interval

FeNO: Fractional exhaled Nitric Oxide

FEV_1: Forced exhaled expiratory volume in the first second

FEV_1/FVC: forced vital capacity ratio

FVC: Forced Vital Capacity

GP: General Practitioner

LLN: Lower Limit of Normality

NPV: Negative Predictive Value

PCC: Primary Care Center

ppb: part per billion

% pred: individualised percentage predicted values

PPV: Positive Predictive Value

ROC: Receiver Operating Characteristic curve

$ULN90^{th}$: Upper Limit for Normality percentile 90

$ULN95^{th}$: Upper Limit for Normality percentile 95

z-score: standardised values for FeNO

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Introduction

Asthma is a chronic and heterogeneous disease, characterised by breath sound patterns and variable expiratory flow limitation, and symptoms such as, dyspnoea or wheezing, that can be mistaken with other respiratory diseases such as chronic obstructive pulmonary disease (COPD), rhinitis and allergies [1]. It affects people of all ages and significantly impacts patients lives, including their health-related quality of life and healthcare utilisation [2]. The global prevalence of asthma varies widely across regions, with approximately 358 million people worldwide suffering from it, a number that tends to increase with urbanisation [3].

In primary care centres (PCC), assessing asthma diagnosis can be challenging. Scarce resources, often due to a lack of diagnostic test [4], such as spirometry and the measurement of biomarkers like the fractional exhaled nitric oxide (FeNO) [5], represent a significant barrier. Although the implementation of this biomarkers can initially be costly and requires a process to disseminate these new practices, they are recognised as cost-effective and respond to the growing demand for personalised asthma care [6]. Given the heterogeneous phenotype and diverse inflammatory mechanisms of asthma, objective biomarkers like FeNO are essential to identify different patterns of the disease and support a more accurate diagnosis [7].

FeNO has emerged as a potentially useful biomarker for assessing airway inflammation and facilitating asthma diagnosis [8, 9]. It is widely recognised as an accurate, reproducible, and non-invasive diagnostic test for respiratory diseases, including COPD, rhinitis, allergies, and asthma [10-12]. This biomarker measures local eosinophilic inflammation in the airways [4] and has traditionally been interpreted using fixed absolute cut-offs. According to the American Thoracic Society (ATS) recommendations, an absolute cut-off above 50 ppb is considered the standardised value for a high probability of local eosinophilic inflammation [13, 14]. Several studies reported that FeNO has an 85% sensitivity and 90% specificity for diagnosing asthma, particularly when combined with the clinical history and in steroid-naive and non-smoking patients [15, 16]. However, current recommendation does not account for

patients' intra-individual factors, such as sex, age, height, smoking habits, and atopy, which strongly influence FeNO levels. To address this need, individualised cut-offs have been proposed [8, 17]. Jacinto et al., developed individualised values using the lambda mu-sigma (LMS) method, which provides more accurate FeNO values, along with upper and lower limits for normality (ULN and LLN, respectively) at the 90^{th} or 95^{th} percentile and the corresponding z-score [10]. This method considers the parameters L, M, and S of the distribution, interpreted as the expected median (M) of a participant, and additionally for the expected coefficient of variation (S) and skewness (L) of a participant [18]. More recently, Amaral et al. [19], obtained robust results by applying this method to derive individualised cut-offs for ruling out an asthma diagnosis. To optimize clinical utility in primary care, two distinct individualized FeNO cut-offs were selected based on their specific diagnostic profiles. The 100% predicted cut-off demonstrated superior sensitivity, reinforcing its clinical value for initial screening and reliably ruling out an asthma diagnosis. Conversely, the 202% predicted cut-off achieved the highest specificity among the personalized values, offering a more robust assessment for ruling in an asthma diagnosis. The application of this two tailored thresholds, thus enhance the diagnostic accuracy and clinical interpretation of FeNO, particularly concerning the identification or exclusion of type 2 inflammation-related airway diseases.

In patients with asthma, FeNO levels are positively correlated with sputum eosinophil counts, the extent of eosinophil infiltration in bronchial biopsies, and bronchoalveolar lavage fluid eosinophil counts [8, 17]. While FeNO and blood eosinophils (B-Eos) are dependent on cytokines related to type 2 inflammation, they reflect different inflammatory pathways and may represent distinct facets of inflammation in asthma [20]. FeNO in patients with asthma can be viewed as a complementary test for asthma diagnosis [21]. Furthermore, in individuals with an elevated eosinophil phenotype, FeNO could have a relevant clinical impact, both in guiding the implementation of therapeutic strategies and in prognostic identification, as a reduction in eosinophils is known to be associated with a decrease in asthma exacerbations and hospitalizations [22, 23].

Eosinophils, immune system cells associated with allergic inflammation, play a fundamental role in asthma. The relationship between peripheral blood eosinophil levels and FeNO levels strengthens the clinical utility of this measurement [14]. The presence of peripheral eosinophilia combined with high FeNO levels increases the likelihood of eosinophilic asthma. Al Ghobain et al., [24] demonstrated that a Blood Eosinophils Count at 300 cells/mm^3 had a sensitivity of 63%, a 77% specificity, and an AUC of 72%, representing an optimal cut-off point for FeNO superior to 25 ppb. Additionally, monitoring eosinophils throughout treatment provides a dynamic approach to asthma management, allowing personalised adjustments to therapeutic strategies [24].

In this study, we hypothesise that FeNO can be used as a screening biomarker tool to rule-out asthma diagnosis in PCC. Our primary aim is to evaluate the diagnostic utility of individualised FeNO values derived from previously developed reference equations. Secondly, we will compare these individualised percentage predicted cut-offs with fixed cut-offs for asthma diagnosis in primary care settings.

Methods

Study Design

This is a secondary analysis of the EPI-ASTHMA study, a population-based, nationwide prevalence study (NCT05169619). EPI-ASTHMA used a stepwise approach with four consecutive stages detailed elsewhere [25]. We used data collected from Stages 1 and 2 collected between May 2021 and March 2024 in 38 PCCs of the Portuguese National Health Service. This PCCs were geographically distributed across all mainland Portugal Health Regions (North, Centre, Lisbon Metropolitan Area, Alentejo, and Algarve) [26]. This study was described according to the Statement for Reporting Studies of Diagnostic Accuracy (STARD) guideline for reporting diagnostic accuracy studies [27].

Ethical considerations

The study protocol was approved by local health research ethics committees of Regional Health Administrations of North (CE/2022/117), Center (27/2021), Lisbon

and Tagus Valley (2775/CES/2022), Alentejo (11/CE/2022), Algarve (1/2022), and of the Local Health Units of Matosinhos (38/CES/JAS) and Alto Minho (38/2021). The study was conducted in accordance with the ethical standards of the Declaration of Helsinki and the ethical recommendations for health research involving human participants. Subjects were invited to participate by clinical secretaries of the PCCs during a phone call (Stage 0), and those providing oral consent were then contacted by a healthcare professional to answer a phone screening interview (Stage 1). Participants with respiratory symptoms during Stage 1 were invited for a diagnostic visit (Stage 2), where written informed consent was obtained prior to all study procedures.

Participants

This secondary analysis included adult subjects who participated in both Stage 1 and Stage 2. Subjects randomly selected from the database of the 38 PCCs were included in Stage 1 if they were at least 18 years old and agreed to answer a screening interview. Subjects reporting respiratory symptoms during Stage 1 were included in Stage 2. Around 5% of subjects with no respiratory symptoms were also invited for Stage 2 for quality control. Subjects with any specific physical and/or cognitive disabilities that prevented them from cooperating with the study procedures (e.g., lung function tests) and/or non-Portuguese speakers were excluded from the study. In this secondary analysis, we additionally excluded subjects who (1) smoked in the last hour, (2) took oral or inhaled corticosteroids in the last 48 hours, (3) exercised vigorously in the last hour, and (4) ate nitrate-rich vegetables, and/or nitrite-rich meats in the last three hours. The first three exclusion criteria seem to underestimate FeNO values [12, 28], and the last one overestimates FeNO levels [8]. We also excluded subjects aged over 80 years and of ethnicity other than Caucasian, as equations for individualised FeNO values have only been calculated using a sample aged between 6 and 80 years and a Caucasian population [10].

Data collection

In this secondary analysis, we used data collected during the diagnostic visit conducted in a mobile unit close to the PCC (Stage 2). The diagnostic visit included clinical history, physical examination, lung function tests (spirometry), inflammatory biomarker testing (FeNO, blood eosinophil count), and patient-reported outcome measures. At the beginning of the visit, sex, age, weight in kilograms, height in centimeters, ethnicity, smoking status, and comorbidities (atopy and eczema) were collected. One general practitioner (GP), clinical physiologist, and clinical researcher were responsible for the data collection. As this was a multicenter study, 179 GPs, 5 clinical physiologists, and 1 clinical researcher were involved.

Spirometry

Lung function variables, such as basal forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), and the ratio between forced expiratory volume in the first second and forced vital capacity (FEV_1/FVC), as well as their predicted values were assessed by spirometry. Spirometry maneuvers followed the ATS recommendations [29], and predictive values were interpreted according to the Global Lung Function Initiative [30]. All measurements were performed using the Minispir S spirometer (MIR, Rome, Italy) and respective software (winspiroPRO, version 8.5.5, MIR, Rome, Italy).

FeNO

A calibration of the FeNO environment was performed before testing. Briefly, participants were asked to place their mouths in the mouthpiece of the analyser and immediately fill their lungs with NO-free air. They were then asked to blow out all the air at a constant rate of 50 ml/s for 10 seconds [31]. They were asked to blow for at least 6 seconds if they could not do so. During the maneuver, participants were given feedback on their performance with the help of an animated display to guide them through the assessment and facilitate collaboration [32]. All FeNO measurements were performed with NIOX VERO equipment (Circassia AB, Uppsala, Sweden) and interpreted using absolute and individualised cut-offs. FeNO values were measured in

part per billion (ppb), while individualised percentage predicted (% pred) refers to the expected normal FeNO value adjusted for individual characteristics, such as age, sex, and height. Absolute cut-offs recommended by the ATS classify FeNO as low, if less than 25 ppb; moderate, between 25-50 ppb; and high, if greater than 50 ppb, which suggests a high probability of local eosinophilic inflammation [13, 14]. FeNO predicted values were obtained for all subjects using lambda-mu-sigma methods with the explanatory variables sex, age, height, and FeNO values obtained during the diagnostic visit at stage 2, using refined prediction equations for FeNO [19]. To establish a better correspondence between sensitivity and specificity and to meet the purpose of our study, different cut-off points were determined, namely 100% predicted and 202% predicted values, to express the likelihood of asthma. We selected the 50 ppb absolute cut-off and the 202% predicted cut-off to rule in asthma, and the 25 ppb absolute cut-off and the 100% predicted cut-off to rule out asthma, respectively.

Blood Eosinophil

A blood sample was obtained by finger prick to measure the blood eosinophil count. All blood eosinophil counts were measured using a point-of-care testing analyser (HemoCue WBC DIFF analyser, HemoCue AB, Angelholm, Sweden). A low probability of systemic eosinophilic inflammation was considered for values below 0.3 µL and a high likelihood of systemic eosinophilic inflammation above 0.3 µL [33].

Statistical analysis

Descriptive statistics were used to characterise sociodemographic and clinical variables. Categorical variables (sex, smoking status, comorbidities, FeNO classification, and blood eosinophil count categories) were presented as absolute frequencies and proportions. Continuous variables (age, body mass index, spirometry measures, FeNO measures) were presented as median and percentiles 25-75. To test differences regarding sociodemographic and clinical characteristics between subjects with asthma and subjects without asthma groups, we used the Mann-Whitney test for continuous data and Chi-Square (X^2) tests for categorical data. When a statistically significant difference was found for a categorical variable with more than two

categories, an X^2 multiple comparison test with Bonferroni correction was performed to explore which categories differed.

To evaluate the discriminative/predictive power of the FeNO measurements, the final asthma diagnose established by the GP was utilised as the reference standard. This diagnosis was determined according to established clinical criteria, encompassing patient history, symptoms, and objective tests (such as spirometry, FeNO and blood eosinophils count), as per international guidelines [13, 30, 34]. Receiver operating characteristic (ROC) curves and the corresponding area under the curve (AUC) analysis were subsequently carried out to quantify the diagnostic performance of the FeNO values against this clinical benchmark. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), were used as diagnostic accuracy measures for 25 ppb and 50 ppb absolute cut-offs proposed by ATS. Additionally, we considered the combination of PPV, NPV, sensitivity, and specificity best suited the purpose of the individualised cut-off points for each case. All statistical analyses were performed using IBM SPSS Statistics (version 29.0) and the R statistical software (version 3.1.1). A p-value ≤ 0.05 was considered statistically significant.

Results

Participants

A total of 1857 subjects participated in Stage 2 of the EPI-ASTHMA study, from which we excluded 474 participants for not meeting the eligibility criteria defined for this secondary analysis (Figure 1).

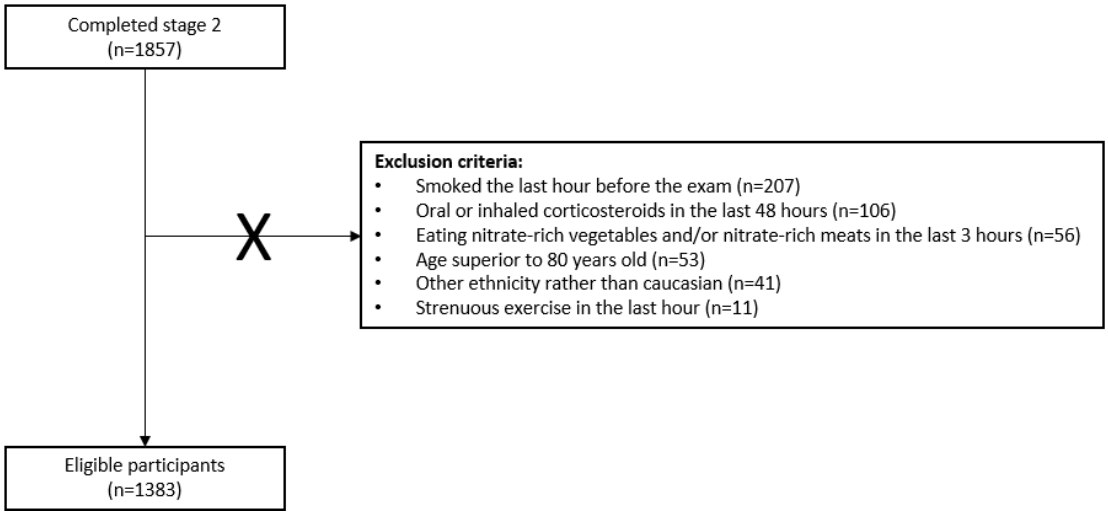

Figure 1: Flowchart describing the selected participants eligible for this secondary analysis.

The characteristics of the included participants are shown in Table 1. Of the 1383 participants included, 833 (60.2%) were female and 258 (18.7%) were diagnosed with asthma. Participants with asthma were younger ($p<0.001$) and had more frequent nasal polyposis ($p=0.001$) and eczema ($p=0.002$) than subjects without asthma. Participants with asthma exhibited significantly elevated FeNO levels (23.0 ppb vs. 16.0 ppb, $p<0.001$), a higher proportion above 50 ppb (22.5% vs. 5.7%, $p<0.001$), and a higher percentage predicted value (115.7% pred vs 102.4% pred, $p<0.001$). Multiple comparisons with the Bonferroni correction, revealed significant differences in FeNO levels between the asthma and non-asthma groups ($p<0.001$). Specifically, there were significant differences between the <25 ppb category ($p=0.0031$) and both the 25–50 ppb and >50 ppb categories ($p < 0.001$).

Blood eosinophil counts differed significantly between the groups ($p<0.001$), being higher in the asthma group than the non asthma group (17.2% vs 8.3%, respectively). The non-asthma group obtained a range of ULN95th and a ULN90th significantly higher ($p<0.001$) than the asthma group; conversely, the z-score showed us a higher significant range in the asthma group compared to the non-asthma group ($p<0.001$) for the FeNO values.

377 **Table 1.** Description of participants included in the study (n=1383).

	Total (n=1383)	Asthma (n=258)	Non-Asthma (n=1125)	p-value
Sex, n (%)				0.102¶
Female	833 (60.2)	167 (64.7)	666 (59.2)	
Age, years	54.0 [43.0-65.0]	49.5 [40.0-61.0]	55.0 [44.0-66.0]	<0.001‡
Height, (cm)	165.0 [159.0-172.0]	165.5 [159.0-171.5]	165.6 [158.0-172.0]	0.918‡
BMI, (kg/m²)	26.9 [24.2-30.7]	27.1 [23.9-30.9]	26.9 [24.2-30.5]	0.795‡
Smoking status[a], n (%)				0.361¶
Never smoker	816 (59.2)	156 (60.5)	660 (59.8)	
Former smoker	401 (29.1)	67 (26.0)	334 (28.9)	
Current smoker	162 (11.7)	35 (13.6)	127 (11.3)	
Comorbidities, n (%)				
Eczema	183 (13.2)	49 (19.0)	134 (11.9)	0.002¶
Nasal polyposis	79 (5.7)	26 (10.1)	53 (4.7)	<0.001¶
Spirometry measurements				
FVC, L	3.33 [2.74-4.01]	3.32 [2.86-4.02]	3.33 [2.70-4.00]	0.213‡
FEV_1, L	2.72 [2.21-3.77]	2.62 [2.22-3.17]	2.74 [2.21-3.31]	0.261‡
FEV_1/FVC	81.6 [77.0-85.4]	78.7 [74.1-83.4]	82.2 [78.1-85.8]	<0.001‡
FeNO measurements				
FeNO, ppb[b]	17.0 [11.0-27.0]	23.0 [13.0-43.7]	16.0 [11.0-25.0]	<0.001‡
FeNO classification[d], n (%)				<0.001¶*
<25.0 ppb	769 (57.8)	105 (43.0)	664 (61.1)	
25.0-50.0 ppb	444 (33.4)	84 (34.4)	360 (33.1)	
>50.0 ppb	117 (8.8)	55 (22.5)	62 (5.7)	
FeNO, % pred[b]	103.9 [88.2-120.9]	115.6 [93.8-143.4]	102.4 [87.4-116.9]	<0.001‡
z-score	0.21 [-0.65,1.16]	0.8 [-0.3-2.2]	0.1 [-0.7-0.9]	<0.001‡
ULN95th[b], ppb	34.6 [30.0-39.7]	32.8 [28.5-38.4]	35.0 [30.3-39.9]	<0.001‡
ULN90th, ppb	29.0 [25.3-33.1]	27.5 [23.9-32.0]	29.5 [25.5-33.3]	<0.001‡
Blood Eosinophils Count[c], n (%)				<0.001¶
≥0.30 µL	124 (9.9)	39 (17.2)	85 (8.3)	

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379 Values are presented as median, M, and percentile 25, p25, and percentile 75, p75, for continuous variables unless
380 otherwise indicated. Abbreviations: BMI, Body Mass Index; cm – centimeters; FeNO, a fraction of exhaled nitric
381 oxide; FEV_1, Forced expiratory volume in the first second; FVC, Forced Vital Capacity; FEV_1/FVC, Tiffenau Index;

Kg/m², kilograms per square meter; L, liters; ppb, parts per billion; %pred, percentage predicted; [a] four missings; [b]53 missings; [c] 133 missings; ¶Tested by Chi-Square test; *multiple comparisons showed that only between 25 ppb and 25.0-50.0 ppb were no statistically significant differences; ‡ Tested by Mann-Whitney U-test; ULN90th, the upper limit for normality percentile 90; ULN95th, the upper limit for normality percentile 95; z-score, standardised values for FeNO.

FeNO as a screening tool

Figure 2 represents, through ROC curves, the ability of FeNO individualised predictive and absolute values to discriminate between participants with and without asthma. The discriminatory capacity for the FeNO absolute value, summarised by the AUC, was 0.63 (95% CI 0.58-0.67), and the AUC for FeNO individualised percentage predicted values was 0.64 (95% CI 0.60-0.69).

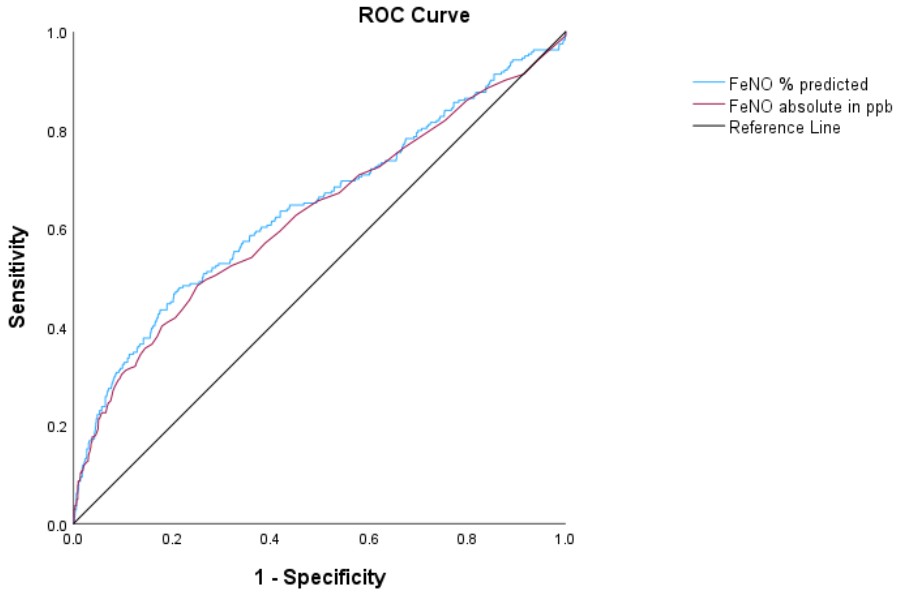

Figure 2: Receiver Operating Characteristics (ROC curve) for FeNO absolute and percentage predicted values.

_____ Reference Line. The solid red line represents FeNO absolute cut-offs, and the solid blue line represents FeNO predictive values. The area under the ROC curve is 0.640 for individualised FeNO values.

Absolute cut-offs according to ATS recommendations

The absolute cut-off of 25 ppb demonstrated moderate sensitivity and specificity for detecting asthma, 57.0% vs 61.1%, respectively. While it achieved a high NPV of 86.4%,

the PPV was considerably lower at 24.8%. The cut-off value of 50 ppb showed inverse characteristics, with low sensitivity (22.5%) but high specificity (94.3%) (Table 2).

Table 2. Discriminative and Predictive Ability of Absolute and Percentage Predicted FeNO Cut-offs.

FeNO measurements	N	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
25 ppb	769	57.0 (50.5-63.3)	61.1 (58.2-64.1)	24.8 (22.4-27.3)	86.4 (84.5-88.0)
50 ppb	117	22.5 (17.5-28.3)	94.3 (92.7-95.6)	47.0 (38.8-55.4)	84.4 (83.4-85.3)
100 % pred	748	68.4 (62.2-74.2)	46.5 (43.5-49.5)	22.3 (20.6-24.1)	86.8 (84.4-88.9)
202 % pred	269	39.8 (33.8-46.2)	84.2 (81.9-86.3)	36.1 (31.5-40.9)	86.2 (84.8-87.4)

Data are presented as n (%) and confidence intervals (95% CI). Abbreviations: FeNO, fractional exhaled nitric oxide; NPV, negative predictive value; ppb, part per billion; % pred, percentage predicted value of FeNO; PPV, positive predictive value.

Individualised percentage predicted cut-off proposed

The cut-off points proposed to assist in ruling in and ruling out asthma diagnosis were 202% predicted and 100% predicted values, respectively. The first cut-off (202% pred) demonstrated low sensitivity but high specificity, 39.8% vs. 84.2%, respectively, resulting in a high NPV of 86.2%. Conversely, the 100% predicted FeNO cut-off exhibited moderate sensitivity and low specificity, 68.4% vs 46.5% compared to the higher cut-off.

Discussion

This secondary analysis represents a significant step in applying FeNO as a diagnostic tool for rule-out asthma. By rigorously validating individualised predicted FeNO values within a large, multicenter PCC population in Portugal, we demonstrate the considerable potential of this approach to improve diagnostic accuracy in this crucial healthcare setting.

As expected and consistent with existing literature, participants with asthma exhibited a significantly higher median FeNO absolute and percentage predicted value to those

without asthma. The overall discriminatory power of FeNO in our study was moderate (0.64).

The use of personalised cut-offs yielded high values of specificity, which aligns and strengthens the findings of Karrasch, Linde, et al [15]. These results underscore the principle that as the cut-off threshold increases, specificity generally improves while sensitivity tends to decrease. Regarding the 100% predicted FeNO cut-off, while it demonstrates improved sensitivity compared to the fixed 25 ppb threshold, a corresponding decrease in specificity was observed. Crucially, both cut-offs maintained high NPV of approximately 86.8% and 86.4%, respectively, which is highly advantageous for ruling out asthma. This high NPV, even with a decrease in specificity for the 100% predicted cut-off, is not contradictory when the primary aim is exclusion, as a high NPV indicates a low probability of the disease given a negative test result. Conversely, both thresholds exhibited similarly low PVP of 22.3% and 24.8%, respectively. When comparing the 202% predicted cut-off with the absolute 50 ppb threshold, all accuracy measures were higher for the absolute cut-off, with the exception of sensitivity (39.8% vs. 22.5%) and the PPV (86.2% vs 84.4%). The narrow confidence intervals observed further reinforce the reliability of these measures. The 202% predicted cut-off, despite some lower accuracy metrics compared to the 50 ppb absolute cut-off, still demonstrates promising results in ruling out asthma due to its high NPV. In contrast, while less sensitive, the absolute cut-offs may be more helpful in confirming asthma in patients with strong clinical suspicion and positive diagnostic findings [15]. However, the lower PPV across all evaluated cut-offs underscores the critical importance of using FeNO in conjunction with other diagnostic tools [35, 36]. Therefore, rather than relying solely on this biomarker for diagnosis, GPs should consider integrating FeNO as part of a comprehensive battery of tests, in combination with other assessments such as spirometry and clinical history.

Limitations and Future Directions

Despite the promising findings, it is essential to acknowledge the limitations of this study. Interpreting FeNO values is inherently complex owing to numerous underlying

causes for variation in levels including: smoking habits, diet, respiratory tract infections, time of day, height, gender and age [37]. While a vital strength of our study lies in its large sample size, recruited from the mainland Portuguese population, its confinement to primary healthcare centres a vital strength may limit the direct extrapolation of results to other clinical settings, such as secondary hospitals. Furthermore, our sample exhibited a notable gender imbalance, with a predominant representation of female subjects (60.2%). This may be attributable to a voluntary bias effect, as feedback from researchers involved in the interview phase (phases 0 and 1 of the original EPI-ASTHMA study), stated that female participants were generally easier to recruit and more willing to engage. Regarding ethnicity, only Caucasian subjects were included in our analysis. This was necessary the fact that the individualised FeNO references equations were developed and validated exclusively for Caucasian populations [10, 19]. Consequently, our findings cannot be directly extrapolated to other ethnic groups, underscoring the relevance of future studies specially addressing this matter. Similarly, the exclusion of participants over the age of 80 from the reference equations used (Jacinto, Amaral et al. 2018, Amaral 2023) poses a challenge for external validation, limiting the generalizability of our results to this older age group. Future research is vital to develop and validate FeNO reference equations that encompass a broader range of ages and ethnicities within the Portuguese population.

Crucially, it is important to address the “gold standard” utilised for asthma diagnosis in our study. Our diagnostic classification was based on the GP clinical assessment, whereas the original studies that established and validated individualised FeNO values typically employed more objective and robust methods, such as methacholine challenge tests or bronchodilator reversibility tests, as their gold standard (Jacinto et al., 2018 and Amaral et al., 2023). This difference in diagnostic reference could potentially influence the reported accuracy of FeNO in our cohort and impact the direct comparability of our findings with those studies. Additionally, factors such as the diurnal variability in FENO [38] were not accounted for in this analysis, which could introduce some variability. Finally, while not a primary aim of this study, the potential influence of comorbidities like atopy and eczema [4] on FeNO levels and its diagnostic

utility represents an essential avenue for subsequent investigation, potentially through refined logistic modelling.

Despite the limitations mentioned above, this study significantly contributes to the existing body of knowledge by being one of the first to rigorously evaluate and validate the performance of individualised FeNO prediction equations within a large, multicentre Portuguese primary care cohort. It provides robust empirical evidence supporting the potential clinical utility of individualised FeNO assessment, particularly highlighting the value of its high NPV for effectively ruling out asthma in a real-world primary care setting. This work lays on the groundwork for potentially refining national healthcare guidelines and standardising asthma diagnosis pathways across Portuguese PCCs. Incorporating individualised FeNO assessment into routine practice could facilitate earlier and more accurate diagnosis, optimise resource allocation by reducing unnecessary specialist referrals, and ultimately enhance patient management strategies. This is particularly relevant for guiding targeted therapies in specific inflammatory phenotypes like eosinophilic asthma, where FeNO can serve as a valuable biomarker [39]. Continued research focused on refining these individualised prediction models and addressing the identified limitations is essential to fully realise the potential of FeNO as a robust diagnostic tool in routine clinical practice.

Conclusion

This secondary analysis provides valuable insights into applying FeNO as a screening tool to rule-out asthma diagnosis within the Portuguese primary care landscape. Our findings corroborate that while FeNO demonstrates moderate overall discriminatory capacity for asthma diagnosis, using individualised percentage-predicted FeNO cut-offs yields distinct advantages, particularly high specificity. Specifically, the 100% and 202% predicted FeNO thresholds exhibited substantial NPVs (approximately 86%), underscoring their utility in effectively ruling out asthma - a crucial function in primary care settings where diagnostic uncertainty often prevails and resources like spirometry may be limited or yield inconclusive results.

Conversely, fixed cut-offs, such as the 50 ppb, retain crucial utility for confirming an asthma diagnosis, particularly in patients with objective supporting evidence (e.g., positive post-bronchodilator spirometry). In contrast, the individualised cut-offs offer unique value in situations of diagnostic uncertainty: the 100% pred cut-off, with its superior sensitivity and consistent NPV, is highly effective for reliably ruling out the diagnosis when clinical history suggests asthma but objective findings are inconclusive. However, the consistently low PPV across all evaluated FeNO thresholds reinforces the critical message that FeNO should not be employed as a standalone diagnostic test. Its interpretation necessitates integration within a comprehensive clinical evaluation, encompassing patient history, symptomatology, and, ideally, spirometry, aligning with previous recommendations [40, 41].

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Conflict of interesting

None

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Reporting Guidelines STARD (The Standards for Reporting of Diagnostic Accuracy Studies)

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	p. 4 "Predicted FeNO levels had a slightly higher discriminatory power (AUC 0.64[95%CI 0.60-0.69])"
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	p. 4 "This is a secondary analysis of the EPI-ASTHMA study. We analysed adult participants randomly recruited from 38 primary healthcare centres in Portugal were analysed."; "Materials and Methods"; "Results"; "Conclusions".
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	p.10 "The diagnosis of asthma in primary care centers (PCC) can be difficult (…) this pathology have heterogeneous phenotype and inflammatory mechanisms (…) fractional exhaled nitric oxide (FeNO), are needed to identify different patterns of asthma and support asthma diagnosis."
	4	Study objectives and hypotheses	p. 10 "We hypothesised that FeNO can be used as an asthma screening biomarker in the PCC. Our primary aim is to evaluate the predictive ability of FeNO to diagnose asthma using individualised FeNO values."
METHODS			
Study design	**5**	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	Not applicable. p. 11 "This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study. EPI-ASTHMA used a stepwise approach with four consecutive stages, which are detailed elsewhere (Jácome *et al*. 2022)."
Participants	**6**	Eligibility criteria	p. 12 "Subjects randomly selected from the database of the 38 PCCs were included in Stage 1 if had at least 18 years old and agreed to answer a screening interview."; p. 13 e 14 "we additionally excluded subjects who (1) smoked in the last hour; (2) took oral or inhaled corticosteroids in the last 48 hours; (3) exercised vigorously in the last hour and (4) ate nitrate-rich vegetables and/or nitrite-rich meats in the last three hours."
	7	On what basis potentially eligible participants were identified	p.12 "Only subjects reporting respiratory symptoms during Stage 1 were included in Stage 2"

		(such as symptoms, results from previous tests, inclusion in registry)	
	8	Where and when potentially eligible participants were identified (setting, location and dates)	p. 12 “Subjects randomly selected from the database of the 38 PCCs were included in Stage 1”
	9	Whether participants formed a consecutive, random or convenience series	p. 12 “Subjects randomly selected from the database of the 38 PCCs were included in Stage 1”
Test methods	**10a**	Index test, in sufficient detail to allow replication	Not applicable. Details about the index test can be found in the original Lambda Mu-Sigma method (Jacinto *el al.* 2018)”
	10b	Reference standard, in sufficient detail to allow replication	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the original Lambda Mu-Sigma method (Jacinto *el al.* 2018)”
	11	Rationale for choosing the reference standard (if alternatives exist)	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the original Lambda Mu-Sigma method (Jacinto *el al.* 2018)”
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	p. 16 “FeNO predicted values were obtained for all subjects using lambda-mu-sigma methods with the explanatory variables sex, age, ethnicity, height and FeNO values obtained during the diagnostic visit at stage 2, using refined prediction equations for FeNO (Amaral 2023). To establish a better correspondence between sensitivity and specificity, and to meet the purpose of our study, different cut-offs points were determined, namely 100% predicted and 202% predicted values, to express the likelihood of asthma”
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	N/A
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	N/A
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	N/A
Analysis	**14**	Methods for estimating or comparing measures of diagnostic accuracy	p. 16 “To evaluate the discriminative/predictive power of the FeNO values, in comparison to the GP final diagnosis of asthma, receiver operating characteristic (ROC) curves analysis was carried out.”
	15	How indeterminate index test or reference standard results were handled	Not applicable. p. 11 “This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study.
	16	How missing data on the index test and reference standard were handled	Not applicable. p. 11 “This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study.

	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	Not applicable. "This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study"
	18	Intended sample size and how it was determined	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the study protocol (Jácome *et al.* 2022)"
RESULTS			
Participants	**19**	Flow of participants, using a diagram	p. 16 Figure 1
	20	Baseline demographic and clinical characteristics of participants	p. 16 "A total of 1857 subjects participated in Stage 2 of EPI-ASTHMA study, from which we excluded 474 participants for not meeting the eligibility criteria defined for this secondary analysis (Figure 1). The characteristics of the included participants are shown in Table 1."
	21a	Distribution of severity of disease in those with the target condition	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the study protocol (Jácome *et al.* 2022)"
	21b	Distribution of alternative diagnoses in those without the target condition	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the study protocol (Jácome *et al.* 2022)"
	22	Time interval and any clinical interventions between index test and reference standard	Not applicable. This study is a secondary analysis of the EPI-ASTHMA. Details about the reference standard can be found in the study protocol (Jácome *et al.* 2022)"
Test results	**23**	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	p. 17 Figure 2
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	p.17 "The discriminatory capacity for the FeNO absolute value, summarised by the AUC was 0.63 (95% CI 0.58-0.67) and the AUC for FeNO individualised percentage predicted values was 0.64 (95% CI 0.60-0.69)."
	25	Any adverse events from performing the index test or the reference standard	Not applicable. "This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study."
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	p.18 "Interpreting FeNO values is not straightforward (…) only used a sample from primary healthcare centers, which may limit the extrapolation of the results to other settings (…)
	27	Implications for practice, including the intended use and clinical role of the index test	p.19 "incorporating FeNO into clinical practice, healthcare providers can make more informed decisions about diagnosis and treatment, ultimately improving patient outcomes. The establishment of individualised predicted FeNO values tailored to the Portuguese population could significantly impact national healthcare policies and the standardization of asthma diagnosis in PCC"

OTHER INFORMATION			
	28	Registration number and name of registry	p.12 “This is a secondary analysis of EPI-ASTHMA study, a population-based, nationwide, prevalence study (NCT05169619).”
	29	Where the full study protocol can be accessed	p. 12 “EPI-ASTHMA used a stepwise approach with four consecutive stages, which are detailed elsewhere (Jácome *et al*. 2022).”
	30	Sources of funding and other support; role of funders	p. 10 “This secondary analysis was not funded, but used data from EPI-ASTHMA, which was sponsored by AstraZeneca”

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STARD 2015

AIM

STARD stands for “Standards for Reporting Diagnostic accuracy studies”. This list of items was developed to contribute to the completeness and transparency of reporting of diagnostic accuracy studies. Authors can use the list to write informative study reports. Editors and peer-reviewers can use it to evaluate whether the information has been included in manuscripts submitted for publication.

EXPLANATION

A **diagnostic accuracy study** evaluates the ability of one or more medical tests to correctly classify study participants as having a **target condition.** This can be a disease, a disease stage, response or benefit from therapy, or an event or condition in the future. A medical test can be an imaging procedure, a laboratory test, elements from history and physical examination, a combination of these, or any other method for collecting information about the current health status of a patient.

The test whose accuracy is evaluated is called **index test.** A study can evaluate the accuracy of one or more index tests. Evaluating the ability of a medical test to correctly classify patients is typically done by comparing the distribution of the index test results with those of the **reference standard**. The reference standard is the best available method for establishing the presence or absence of the target condition. An accuracy study can rely on one or more reference standards.

If test results are categorised as either positive or negative, the cross tabulation of the index test results against those of the reference standard can be used to estimate the **sensitivity** of the index test (the proportion of participants *with* the target condition who have a positive index test), and its **specificity** (the proportion *without* the target condition who have a negative index test). From this cross tabulation (sometimes referred to as the contingency or “2x2” table), several other accuracy statistics can be estimated, such as the positive and negative **predictive values** of the test. Confidence intervals around estimates of accuracy can then be calculated to quantify the statistical **precision** of the measurements.

If the index test results can take more than two values, categorization of test results as positive or negative requires a **test positivity cut-off**. When multiple such cut-offs can be defined, authors can report a receiver operating characteristic (ROC) curve which graphically represents the combination of sensitivity and specificity for each possible test positivity cut-off. The **area under the ROC curve** informs in a single numerical value about the overall diagnostic accuracy of the index test.

The **intended use** of a medical test can be diagnosis, screening, staging, monitoring, surveillance, prediction or prognosis. The **clinical role** of a test explains its position relative to existing tests in the clinical pathway. A replacement test, for example, replaces an existing test. A triage test is used before an existing test; an add-on test is used after an existing test.

Besides diagnostic accuracy, several other outcomes and statistics may be relevant in the evaluation of medical tests. Medical tests can also be used to classify patients for purposes other than diagnosis, such as staging or prognosis. The STARD list was not explicitly developed for these other outcomes, statistics, and study types, although most STARD items would still apply.

DEVELOPMENT

This STARD list was released in 2015. The 30 items were identified by an international expert group of methodologists, researchers, and editors. The guiding principle in the development of STARD was to select items that, when reported, would help readers to judge the potential for bias in the study, to appraise the applicability of the study findings and the validity of conclusions and recommendations. The list represents an update of the first version, which was published in 2003.

More information can be found on http://www.equator-network.org/reporting-guidelines/stard.

Regras de formatação e normas da revista: Pulmonology

Article structure

Subdivision - unnumbered sections

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarised, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title*.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations*.** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author*.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address*.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For

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5. Cancer Research UK. Cancer statistics reports for the UK, http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/; 2003 [accessed 13 March 2003].

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[dataset] 6. Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. https://doi.org/10.17632/xwj98nb39r.1.

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ANNEX 1

Literature Review Summary Table

Utility of Individualised FeNO in Diagnosing Asthma in Primary Care Settings

Author(s) & Year	Study Focus / Type	Key Findings Related to FeNO & Diagnosis	Relevance to Individualised FeNO in Primary Care
General Consensus / Multiple Refs (e.g., Taylor 2006; Jacinto et al. 2018; Karrasch et al. 2017)	Reviews & Foundational Studies on FeNO	FeNO is a validated, non-invasive biomarker of eosinophilic airway inflammation. It shows moderate-to-good diagnostic accuracy for asthma, especially certain phenotypes (e.g., allergic/eosinophilic). Accuracy varies with cut-offs and patient populations.	Establishes FeNO as a potentially useful tool for asthma diagnosis, which could be valuable in primary care if interpreted correctly.
ATS (2023) / Dweik et al. (2011)	Guideline Development	Recommended standardised methods for FeNO measurement and provided interpretation guidelines based on fixed absolute cut-off values (e.g., <25 ppb low, >50 ppb high likelihood of eosinophilic inflammation/steroid response).	Provides the basis for the commonly used 'fixed cut-off' approach. Highlights a standard practice against which individualised approaches can be compared.
Bjermer et al. (2014); Matsunaga et al. (2021) & others	Research / Reviews on FeNO Interpretation	Explicitly identified limitations of fixed FeNO cut-offs, noting significant influence from individual factors (age, sex, height, atopy, smoking). Proposed using individualised reference equations or predicted values for more accurate interpretation.	Directly advocates for moving beyond fixed cut-offs to individualised values, suggesting this approach could improve diagnostic accuracy by accounting for patient-specific variations, which is crucial in diverse primary care populations.

Karrasch et al. (2017)	Systematic Review & Meta-analysis (Diagnostic Accuracy)	Confirmed FeNO diagnostic value but emphasised heterogeneity in accuracy across studies. Performance is impacted by factors like smoking, steroid use, sex, height, age, and chosen cut-off.	Underscores how FeNO is interpreted (i.e., the cut-off strategy) significantly impacts its diagnostic utility. This support investigating potentially superior interpretation strategies like individualization, especially for heterogeneous primary care groups.
Barański & Schlünssen (2022); H. Sharpe (2020)	Studies/Commentary on Primary Care Diagnosis	Highlighted diagnostic challenges in primary care (resource scarcity, lack of specialised tests like spirometry/FeNO). Asthma diagnosis often relies heavily on symptoms, leading to potential misdiagnosis.	Emphasises the need for accessible and accurate diagnostic aids in primary care. If individualised FeNO proves more accurate than fixed cut-offs, it could be a more effective tool to overcome some of these diagnostic hurdles in the PCC setting.

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