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NO_x in exhaled breath condensate is related to allergic sensitization in young and middle-aged adults

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EBC NO_x in adults with different allergic phenotypes.

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Abbreviations:

EBC:	Exhaled breath condensate.	SPT:	Skin prick test.
NO:	Nitric oxide.	ICS:	Inhaled corticosteroid.
NO_x:	Total nitric oxide products.	OR:	Odds ratio.
BHR:	Bronchial hyperresponsiveness.	β:	Regression coefficients.
FeNO:	Fractional exhaled nitric oxide.	HDM:	House dust mite.
MACS:	Melbourne Atopy Cohort Study.	BD:	Bronchodilator.
TAHS:	Tasmanian Longitudinal Health Study.	FEV_1:	Forced expiratory volume in the first second.
I^2:	Test of heterogeneity in meta-analysis.	FVC:	Forced vital capacity.

Abstract

Background: Asthma and allergic diseases are heterogeneous. Measurement of biomarkers in exhaled breath condensate (EBC) may help to discriminate between different phenotypes and may assist with clinical prognostication.

Objectives: We aimed to assess associations between total nitric oxide products (NO_x) in EBC and different allergic phenotypes and lung function in young and middle-aged adults.

Methods: Cross-sectional analyses were nested within two Australian longitudinal studies, the Melbourne Atopy Cohort Study (MACS, mean age 17.8 years) and the Tasmanian Longitudinal Health Study (TAHS, mean age 49.4 years). Levels of EBC NO_x were determined by Griess-reaction fluorescent method. Associations were assessed between EBC NO_x and different allergic phenotypes, lung function and airway reactivity.

Results: Atopy, with or without asthma or rhinitis, was associated with increased EBC NO_x levels particularly in individuals with poly- aero-sensitization. These findings were generally consistent across the two age groups. In the older cohort, use of ICS in the previous 12 months masked the association between sensitization and EBC NO_x (OR=0.64, 95%CI=0.21-1.96, p for interaction=0.05).

Conclusions & clinical relevance: In these population-based samples, EBC NO_x was most strongly associated with atopic sensitization, rather than either current asthma or rhinitis, possibly indicating underlying increased airway inflammation associated with atopy. Therefore, EBC NO_x could be a key predictor of atopy in both young and middle-aged adults, regardless of the presence of concomitant asthma or rhinitis.

Key words: Atopy; Asthma; Rhinitis; Exhaled breath condensate; Total nitric oxide.

Introduction

There is increasing recognition that asthma is a heterogeneous condition, with multiple phenotypes (1). The patterns of symptoms and associations with risk factors are likely to be different between these phenotypes, reflecting differences in aetiology, manifestations and the underlying pathophysiology (2). Accurate characterization of these phenotypes may enable researchers to identify potential factors that predict the risk of developing specific forms of asthma and their prognosis in the long-term. An assessment of airway biomarkers of adults with asthma may assist in differentiating between phenotypes (3). The usefulness of oxidative stress biomarkers in the exhaled breath condensate (EBC) in this manner is a current topic of interest.

Nitric oxide (NO) is a biological mediator of eosinophilic (4) and neutrophilic inflammation (5), and has been implicated in the physiological regulation of the airways and pathophysiology of airway diseases (4, 6). NO is enzymatically generated by nitric oxide synthase, then oxidized to nitrite and nitrate by several mechanisms, including macrophage activation (7). Total nitric oxide products (NO_x, nitrate and nitrite) are the more stable end-products of nitric oxide metabolism and can be detected in EBC (8, 9). Recently, anti-IL-13 treatment has suggested that IL-13 is the predominant pathway affecting NO generation rather than eosinophils alone (10). EBC samples the entire respiratory tract from the mouth to the alveoli (7) and as such, the precise airway origin of each EBC marker is unknown (11). Although many of the inflammatory markers are likely to come from the lower airways, some will also be generated from the larger and upper airways (12). Several studies have examined the associations between oxidative stress biomarkers in EBC and asthma (13, 14), but less is known about the associations between the levels of EBC biomarkers and rhinitis

(15). Moreover, atopic sensitization is strongly associated as a prelude to the development of allergic diseases, including asthma and rhinitis, which has given rise to the concept of the "unified airway". However similar clinical allergic features can be initiated by different immune mechanisms (16), and so it remains unclear whether NO-related biomarkers in EBC are uniformly associated with the presence and patterns of atopic sensitization.

EBC total nitric oxide products (NO_x) are increased in individuals with asthma compared to healthy individuals (17, 18). Previous studies have had insufficient power to compare associations between allergic disease phenotypes and EBC NO_x. In addition, most studies have been conducted in clinical populations with more severe disease, rather than population-based samples with a less severe spectrum of asthma, and likely very few truly severely affected individuals. Associations between inflammatory markers and asthma phenotypes may change with age as environmental triggers become increasingly important in later life (7).

It remains possible that the associations with EBC biomarkers may change with age, but this has not been examined in a single study using the same methods and we hypothesized that EBC NO_x would be associated with all subsets of atopic disease. Therefore, using data from two prospective cohorts, we aimed to investigate the associations between the levels of NO_x in EBC and: (1) different allergic phenotypes of asthma, atopic sensitization and rhinitis; (2) lung function, bronchial hyper-responsiveness (BHR) and fraction of exhaled nitric oxide (FeNO); and (3) to assess if these associations were consistent in young and middle-aged adults.

Methods

Study Population

Participants from two Australian longitudinal studies with different age groups were included (1) the Melbourne Atopy Cohort Study (MACS) (n=424, mean (±SD) age: 17.8 ± 1.2 years, described throughout as the young group) (19) and (2) the Tasmanian Longitudinal Health Study (TAHS) (n=794, mean age: 49.4 ± 0.5 years, described as the middle-aged group) (20). The MACS is a study of individuals with a family history of allergic diseases whereas TAHS is a whole population study. The recruitment process and protocol for both studies have been described elsewhere (19, 20). See **e-Appendix 1** in the online data supplement for further details.

The MACS was initially approved by the Mercy Maternity Hospital Ethics Committee (HREC no: R07/20 and R88/06) and thereafter by Royal Children's Ethics Committee (HREC: 28035). The TAHS was approved by the Human Ethics Review Committees at The Universities of Melbourne (approval number 040375), Tasmania (040375.1) and New South Wales (08094), the Alfred Hospital (1118/04), and Royal Brisbane & Women's Hospital Health Service District (2006/037).

Collection of EBC

EBC samples were collected from participants by following ATS/ERS protocols using an established method (21, 22). After oral lavage, participants breathed tidally into a glass condenser via a one-way valve with saliva trap. EBC was collected using a custom-made glass-condensing device containing a thermal flask chilled with ice. Collection took between 15 and 20 minutes to obtain 0.4-3.0 ml of EBC. Each sample was de-aerated with argon gas for 20 seconds, and then

immediately frozen and stored at –80°C for later analysis. Storage times of the collected EBC samples were different between participants within each study and also between the two cohorts, which varied from 3.9 to 4.7 years, (see **e-Table 1** in the online data supplement).

Analysis of total nitric oxide products (NO_x)

NO_x concentration in EBC was measured after enzymatic reduction of nitrate using a fluorescent modification of the Griess method (23). The absorbance of duplicate standards of $NaNO_2$ and thawed EBC samples was measured at excitation 360/40 nm, emission 395/25 nm, and gain 50, CRU (FlexStation-II fluorescent plate reader, Molecular Devices Corporation, 1311 Orleans Drive, Sunnyvale, CA 94089, USA). NO_x concentrations were estimated from the standard curve by interpolation, and the overall %CV of the within-individual reliability for the duplicate values was obtained. The limit of detection (LOD) of the NO_x assay was 2.50 μM.

The outcome measures including skin prick tests (SPTs), lung function testing, bronchial hyperresponsiveness to inhaled methacholine (BHR) (in TAHS only) and fractional exhaled nitric oxide (FeNO) (in MACS only) measurement were taken using standardized techniques (see **e-Appendix 1** in the online data supplement for details).

See **e-Appendix 1** in the online data supplement for the definitions used in this manuscript.

Statistical methods

We found that about half of the participants had readings below the lower LOD (<2.50 μM) for EBC NO_x. Rather than exclude these participants, non-

detectable values of EBC NO_x were assigned to equal half the detection level (=1.25 µM) (24). The distribution of EBC NO_x concentrations was highly right skewed in both cohorts and could not be transformed to approximate normality. Therefore, cut-point levels of low (=1.25 µM), medium (1.26-19.9 µM) and high (≥20.0 µM) were selected, and ordinal logistic regression models were used to assess the associations between various allergic phenotypes (based on symptoms in the previous 12 months) as exposures and levels of EBC NO_x as outcomes. All models met the proportional odds assumption for both overall group and subgroups and the parallel regression assumption, as assessed using the Brant test. Potential confounders were selected on the basis of current knowledge and/or biological plausibility. Some studies have suggested that levels of EBC biomarkers are directly associated with sex and age (25, 26) and these variables should be considered as confounding factors when interpreting the results (11). The duration of EBC sample storage between the sample collecting and analyses was also considered as a potential confounder in this study. The associations between clinical conditions and EBC NO_x have been reported as odds ratios (OR) with corresponding 95% confidence intervals (CI), and the associations between levels of EBC NO_x and markers of airway inflammation including lung function, BHR, and FeNO reported as regression coefficients (β) with corresponding 95%CI.

Potential effect modification between the exposure of interest and potential modifier was examined by testing an interaction term in the model. Likelihood ratio tests were used to test for possible interactions between each exposure of interest and the following variables: sex, history of recent smoking, BMI and any use of ICS in the last 12 months. Strata specific associations were reported if the interaction p-value was less than 0.10.

Meta-analyses were conducted in order to obtain the pooled estimates of associations across the MACS and TAHS cohorts when the heterogeneity of associations, as assessed by I^2 statistic, was less than 80% (27). A fixed-effect model was used when the I^2 statistic was less than or equal to 30% and a random-effect model used when I^2 was more than 30%. All data were analysed using Stata software package, version 13.1 (StataCorp, College Station, Texas).

Results

Characteristics of the study populations

In the MACS, 85.1% (n=361) of participants provided an EBC sample at the 18-year follow-up compared to 56.9% (n=452) of participants at the 5th-decade TAHS follow-up.

There was approximately an even sex-distribution in both cohorts **(Table 1**). As expected due to the inclusion criteria, the MACS cohort was younger and had more allergic disease than the TAHS cohort. Thus, the rate of atopic sensitization (and aero-sensitization) was higher in the MACS than the TAHS (67% vs. 57%). Sensitization to house dust mite (HDM) was common in both cohorts, particularly MACS (63% vs. 48% for TAHS). The proportion of participants who used ICS medication in the TAHS (12%) was slightly greater than that in the MACS (7%), as was current or ever having had rhinitis.

Overall description of the EBC NO_x values

Approximately half of the samples were above the lower limit of detection in each study (MACS 48%, TAHS 54%, see **e-Figure 1** & **e-Figure 2** in the online data supplement). Additionally, there were negative associations between the levels of EBC NO_x and duration of sample storage in both cohorts (see **e-Figure 3** & **e-Figure 4** in the online data supplement). There was no significant difference in the median values of EBC NO_x between the TAHS and MACS populations (p=0.16).

Associations between current asthma, asthma phenotypes and EBC NO_x

While current asthma in each cohort was associated with a non-significant trend for elevated levels of EBC $NO_{x,}$ when these results were pooled using meta-analysis, the odds of elevated EBC NO_x level in participants with current asthma were significantly increased (OR =1.57 [95%CI: 1.02,2.13], **Table 2**). EBC NO_x values were higher in participants with current atopic asthma compared to the reference group with neither asthma nor atopy (**Table 2**). Although there was no evidence of elevated levels of EBC NO_x in non-atopic asthmatic participants, there were limited numbers of participants with this phenotype in both studies. There was an association between NO_x and the atopic non-asthmatic phenotype in the TAHS cohort, but not the MACS cohort (I^2=71%, p-value of heterogeneity =0.06, **Table 2**).

Association between atopic sensitization and EBC NO_x

In both cohorts, the odds of an elevated EBC NO_x level were greater in those with atopic sensitization compared to those without atopic sensitization. The association was somewhat stronger in the TAHS than MACS (p-value of heterogeneity =0.22) (see **e-Table 2** in the online data supplement). This association was stronger in those with poly- aero-sensitization.

The associations between mono- aero-sensitization and levels of EBC NO_x were substantially different between the two cohorts. In the TAHS, the odds of an elevated EBC NO_x level were approximately 2.2-fold greater in participants with mono- aero-sensitization, when compared to those without atopic sensitization. In contrast, in the MACS cohort, there was no evidence of a significant association between mono-aero-sensitization and higher levels of NO_x (p=0.85). These differences did not appear to be due to the type of specific aeroallergens, since the vast majority of mono-sensitized participants in both studies were sensitized to HDM only (see **e-Table 3** in the online

data supplement). Interestingly, the median wheal size to house dust mite in MACS mono-sensitized participants was greater (median: 5 mm [IQR: 4-7]) than observed in mono-sensitized participants in TAHS (3.5 mm [1-5.5], p<0.01), despite mono-sensitization not being associated with NO_x in the MACS.

In both the MACS and TAHS cohorts, we saw an approximately 3% increase in odds for every 1 mm in the sum of the SPT wheals (for both MACS and TAHS OR=1.03, 95%CI=1.01-1.05).

Associations between current rhinitis, rhinitis phenotypes and EBC NO_x

EBC NO_x was associated with current rhinitis in TAHS (**Table 3**), but not in MACS. Furthermore, in the TAHS only, the odds of an elevated EBC NO_x level were higher in those participants with atopic non-rhinitis, and also in those with atopic rhinitis, when compared to participants with neither atopy nor rhinitis. These associations were not observed in the MACS and there was evidence that the associations differed between the two cohorts (I^2=70%) (**Table 3**).

Factors associated with EBC NO_x levels

To identify independent factors associated with EBC NO_x, we performed mutually adjusted regression models (adjusting for all variables under investigation). In both cohorts, the odds of an elevated EBC NO_x level were greater in participants with poly-aero-sensitization (see **e-Table 4** in the online data supplement). In the TAHS only, the odds of elevated EBC NO_x level were greater in participants with mono-aero-sensitization, and there was evidence that this association differed between the two cohorts (I^2=65%) (see **e-Table 4** in the online data supplement).

There were no associations with the other factors assessed such as smoking history or the use of any ICS in the previous 12 months.

To further explore any subgroup associations, all two-way interactions between each factor listed in **e-Table 4** were assessed using likelihood ratio tests. The majority of these interactions were not statistically significant ($p>0.10$) with few interactions being present only in the TAHS (**Table 4)**. There was evidence of an interaction between the status of atopic sensitization and current use of ICS medication in the TAHS cohort. The odds of an elevated EBC NO_x level were approximately 2.9-fold higher in sensitized participants who did not use any ICS medications in the previous 12 months. In contrast, atopy was not associated with NO_x in those who were currently taking ICS. Moreover, sex modified the association between atopic sensitization and the level of EBC NO_x. In males, the odds of an elevated EBC NO_x level were approximately 4.4-fold higher in sensitized than non-sensitized participants. There was no association seen between atopy and NO_x in females.

EBC NO_x and lung function, BHR and FeNO

In the MACS cohort, the pre-bronchodilator (BD) FEV_1/FVC ratio decreased by 7% for each 1 μM increase in EBC NO_x (95%CI: reduced from 1 to 13%, $p=0.04$, see **e-Table 5** in the online data supplement), while FEV_1 was not associated with NO_x. These associations were essentially the same when post-BD lung function was assessed. In the TAHS cohort, there were no associations between increasing EBC NO_x and any pre-BD lung function parameters, while post-BD spirometric measures were not performed as BHR was assessed. There was no evidence that sex, history of recent smoking, BMI, current asthma or any use of ICS in the previous 12 months modified these associations.

There was no evidence of an association between EBC NO_x levels and a positive BHR (OR=1.01[95%CI: 0.99,1.03], p=0.50) or with log dose response (β: -0.002 [95%CI: -0.01,0.1], p=0.67) in the TAHS cohort. While there was a weak trend in the MACS cohort towards increased FeNO being associated with increasing levels of EBC NO_x (β: 0.41 [95%CI: -0.01,0.82], p=0.05, **e-Figure 5** in the online data supplement), there was evidence that this association was modified by aero allergen sensitization status (p<0.01). While there was no evidence of an association in those with no or mono-sensitization, the association was stronger in those with poly-sensitization (β: 0.73 [95%CI: 0.23,0.1.23], p<0.01).

Discussion

In this study of two adult cohorts of different ages, we found there was an association between atopy and higher levels of EBC NO_x in both age groups, regardless of concurrent asthma or rhinitis. This association was stronger in those with poly-aero-sensitization in both cohorts. Increased levels of EBC NO_x were related to decreased pre- and post-BD FEV_1/FVC only in the young adults (MACS). There was no evidence of any significant association between increased concentrations of NO_x and BHR. Most of the observed associations were consistent between young and middle-aged groups.

In both age groups, higher levels of EBC NO_x were found in participants who had atopic sensitization. The underlying pathophysiology of atopic sensitization involves the activation of inflammatory cells and the release of inflammatory mediators (28). In response to specific allergens, both airway epithelial cells and dendritic cells are stimulated to release various cytokines and thymic stromal lymphopoietin which activates mast cells, eosinophils and T_H2 cells in sensitized individuals (28, 29). These mediators promote eosinophil infiltration and proliferation in the airway mucosa, mast cell degranulation and airway hyper-responsiveness (30). Eosinophilic, IL-13 and IL-4 related airway inflammation are recognized as causing increased NO generation, elevated FeNO and hence increases the concentration of reactive-nitrogen species and their potential contribution to oxidative stress (31).

We found that poly-aero-sensitization was associated with higher levels of EBC NO_x, in both age groups. These findings suggest that an increased level of EBC NO_x may be considered as a biological marker for the intensity of atopic sensitization in both young and middle-aged adults. It has been shown previously that sensitization to more

than one allergen is associated with an increased risk of having asthma (32). Interestingly, in the middle-aged group, this association was not seen in those who had used ICS in the previous 12 months, possibly indicating active suppression of NO_x production.

The associations between current asthma or current rhinitis and EBC NO_x were not as clear or as strong as those seen for sensitization in the combined data from both age groups. Although we had limited numbers of participants with asthma or rhinitis but without atopy, there was no evidence of elevated levels of NO_x in these participants overall. This pattern of results suggests the most robust association between EBC NO_x and asthma and rhinitis is predominantly due to the co-occurrence of allergic sensitization.

Interestingly, we found that elevated EBC NO_x levels were associated with atopic non-asthma in the TAHS participants. It is possible that some of these participants had prior asthma leading to airway remodeling (33), ongoing airway inflammation, and elevated oxidative stress (34, 35) but quite possibly, atopy may directly influence NO_x, without requiring asthma to be intermediary step.

There was a significant association with allergic rhinitis and EBC NO_x found in the TAHS cohort only. In contrast, non-atopic rhinitis showed no significant associations with the levels of EBC NO_x in either study. It remains unclear why this is the case, and may be due to the younger age of participants in MACS, or that all participants in this study have a family history of allergic disease.

The association between current asthma and EBC NO_x observed in this study is relatively weak, when compared to previous studies (18, 36, 37). This could be due to differences in the study populations or in the definitions of asthma, with others mostly

studying more severe asthma. In previous studies, asthma was mainly defined according to clinical manifestations, physician diagnosis, responsiveness to bronchodilator drugs, and/or measurements of pulmonary function (15, 38). In the current study, in contrast, asthma was defined according to the questionnaire responses by the participants. Moreover, the use of different collecting devices to sample EBC, or different analytical assays to measure NO_x levels may also have created differences between studies. The type of collecting device clearly influences the levels of many EBC biomarkers, either by the amount of water vapour condensed, or the materials used in the condenser (39, 40). In addition, the Griess method of measuring NO_x may be limited in biological systems, and electrochemical methods may provide a more rapid method of analysis (41). In epidemiological studies, collecting EBC at field sites may be more convenient and more practical than having NO analysis acutely available (40, 42).

Another recent study reported median values of ~ 7.2 µM (43) in middle aged participants without respiratory disease, while the values we see in this study are lower (~1.5 µM). This could be due to a range of factors, including the due to the storage resulting in reduced measurable levels, plus the different device used (e.g. glass condenser versus R-tube or other devices), and ambient pollution levels.

In this study, EBC NO_x was influenced by the long period of storage. Despite being frozen at −80 °C, it is likely that biomarkers were degraded during the storage period and so the duration of sample storage needs to be considered when analysing EBC biomarkers in future studies. A challenge for epidemiological studies, where large numbers of participants are recruited, is that recruitment takes time to complete, and

rapid sample analysis would require multiple batches, which are costly and can itself cause errors due to batch-to-batch variation.

Additional longitudinal studies are required, where EBC biomarkers are measured repeatedly in the same participants, to determine if changes in these biomarkers could predict subsequent exacerbations of airway inflammation, and to determine the impact of other factors, such as smoking and air pollution exposures. If a spike in the levels of specific biomarkers occurs prior to clinical symptoms, this may open avenues to the use of EBC as a treatment-modifying test.

In young adults only, an increased EBC NO_x level was associated with a reduction in the pre- and post-BD FEV_1/FVC ratio, but not with other lung function measurements. Several studies have demonstrated negative correlations between EBC NO_x and other spirometric variables, such as FEV_1 (18, 44) and peak expiratory flow (18) which could be due to the EBC NO_x level reflecting ongoing inflammatory airway obstruction (45).

Strengths & limitations

The main strength of this study is that the associations were investigated within two large longitudinal cohorts. A large number of EBC samples from these cohorts were collected, which made it possible to compare and contrast the findings. In addition, the available data combined from both cohorts were used to identify different phenotypes of allergic airway diseases in order to evaluate the levels of EBC biomarkers in each phenotypic subgroup regardless of age. Both the questionnaires and the objective measures (such as SPT and lung function) were standardized across studies and all questions were validated.

This study also has a number of limitations. In both cohorts, approximately half of the samples analyzed for NO_x were less than the lower limit of detection. In addition, there was a non-linear, negative relationship between EBC NO_x and duration of storage. This suggests that long periods of storage of EBC samples may reduce the measured concentrations of EBC NO_x. To account for this, we have adjusted for the duration of storage for all analyses. A more sensitive assay, which allowed for quantification of NO_x in a higher proportion of participants, may have allowed for a clearer association between NO_x and FeNO to have been observed in the non and mono-sensitized groups.

While the results are based on prospective cohorts, these analyses are cross-sectional in nature, and this design gives no indication of the sequence of events. While we were able to assess associations with use of inhaled steroids in the previous 12 months, we did not have records of the time since last use, which should be explored in future. The MACS is a younger, high-risk allergy cohort while the TAHS is a population-based cohort. There may be risk factors and pathways in the development of allergic disease and airway inflammation that are different between high- and normal-risk populations, which may explain some of the differences seen between the two cohorts. However, with some exceptions the associations seen were largely similar between the two cohorts. Although both cohorts were conducted in Australia, the populations were recruited from different regions. The MACS participants represent young adults from an urban area (Melbourne), whereas the TAHS participants represent middle-aged adults from less urbanized area with a greater proportion living in a rural setting (Tasmania). Each population, therefore, was exposed to differing environmental factors such as air pollution, allergens, infection,

hygiene and diet. These differences in exposure to environment could possibly influence the observed differences between the two cohorts.

Validated questionnaires were used in both cohorts for defining current asthma, but lack of an objective definition is a potential limitation of this study, as is a lack of a consistent measure of asthma severity that could be used in both studies.

In conclusion, the current study demonstrates that atopy, rather than asthma or rhinitis, plays a key role in determining increased levels of EBC NO_x. As this association was masked in the older participants who had ICS in the previous 12 months, it is also important to consider the prior use of medication. EBC is a non-invasive method that holds promise for detecting markers of inflammation that could be used to detect atopic diseases, and perhaps be able to monitor the effects of inhaled medication. Further studies are also needed, including the establishment of appropriate standardization and reference values for biomarkers in EBC.

Conflict of interest

M.J.A holds investigator initiated grants from Pfizer and Boehringer-Ingelheim for unrelated research. He has also received assistance with conference attendance from Sanofi. The other authors declare they have no conflict of interest in relation to this manuscript.

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Author contributions

S.C.D, A.J.L, M.C.M, C.J.L, M.J.A, and P.S.T designed the 18-year MACS follow-up and S.C.D, M.C.M, M.J.A, E.H.W and P.S.T designed the 45-year TAHS follow-up, obtained funds and conducted the studies from which the data and biospecimens have been used for this manuscript. F.M.A analyzed the EBC samples and performed the statistical analyses with input from A.J.L, P.S.T, S.C.D, J.E.B and M.C.M and wrote the first draft of the manuscript. All authors critically revised and edited the manuscript and approved the final version.

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Table 1: Characteristics of participants in MACS and TAHS cohorts.

	MACS (n=361)*	TAHS (n=452)*	P-value
Males	50.4 (182)	50.4 (228)	0.10
Age in years; Mean (SD)	17.8 (1.2)	49.4 (0.5)	**<0.01**
Ever smoked†	7.7 (27)	55.8 (252)	**<0.01**
Recent smoking	4.8 (17)	15.1 (68)	**<0.01**
Atopic sensitization	67.0 (235)	56.7 (251)	**0.01**
Food sensitization	12.0 (42)	NA	-
Aero-sensitization	66.4 (233)	56.7 (251)	**0.01**
Mono- aero-sensitization	15.4 (54)	18.1 (80)	
Poly- aero-sensitization	51.0 (179)	38.6 (171)	
HDM	55.3 (194)	40.1 (179)	**<0.01**
Cat hair	25.6 (90)	17.6 (78)	**0.01**
Mixed grasses	40.7 (143)	33.2 (147)	**0.01**
Ryegrass	43.6 (153)	33.0 (146)	**0.01**
Alternaria	12.8 (45)	7.2 (32)	**<0.01**
Penicillium	4.0 (14)	2.5 (11)	**0.07**
Hormodendrum	NA	3.6 (16)	-
Aspergillus fumigatus	NA	2.3 (10)	-
Asthma			
Ever asthma	40.5 (142)	36.1 (162)	0.21
Current asthma	25.3 (86)	19.6 (88)	0.06
Any use of ICS medication	6.9 (22)	12.1 (54)	**0.02**
Rhinitis			
Ever rhinitis	49.3 (175)	62.1 (280)	**<0.01**
Current rhinitis	36.8 (131)	60.4 (256)	**<0.01**

* **All data are presented in % (n) unless specified.**

† **Ever smoked defined as having smoked ≥100 cigarettes in their life, while recent smoking defined as having smoked in the 24 hours prior to testing.**

Significant P-values of less than 0.05 are bolded.

Abbreviations: **MACS**: Melbourne Atopy Cohort Study; **TAHS**: Tasmanian Longitudinal Health Study; **SD**: Standard deviation; **HDM**: House dust mite; **ICS**: Inhaled corticosteroids; **NA**: Not assessed.

Table 2: Associations between asthma, asthma phenotypes and levels of EBC NO_x.

	MACS (n=340)				TAHS (n=449)				Meta-analysis	
	% (n)	NO_x (µM) Median [IQR]	OR* [95%CI]	P-value†	% (n)	NO_x (µM) Median [IQR]	OR* [95%CI]	P-value†	Pooled Estimate [95%CI]	I^2 (%)
Current asthma										
NO	74.7 (254)	1.25 [1.25-4.54]	1.00	-	80.4 (361)	2.58 [1.25-4.38]	1.00	-	1.00	-
YES	25.3 (86)	2.64 [1.25-5.18]	1.61 [0.98, 2.65]	0.06	19.6 (88)	2.97 [1.25-4.67]	1.54 [0.97, 2.45]	0.07	**1.57 [1.02, 2.13]**	0
Asthma phenotypes (MACS: n‡ =331, n: n‡ =440)										
Non-atopic non-asthmatics	31.1 (103)	1.25 [1.25-3.68]	1.00	-	37.3 (164)	1.25 [1.25-3.11]	1.00	-	1.00	-
Atopic non-asthmatics	44.1 (146)	1.25 [1.25-4.90]	1.40 [0.81, 2.42]	0.22	43.2 (190)	3.27 [1.25-6.88]	**2.79 [1.83, 4.26]**	**<0.01**	2.02 [0.67, 3.37]	71
Non-atopic asthmatics	3.0 (10)	1.25 [1.25-4.35]	1.61 [0.45, 5.82]	0.47	5.7 (25)	3.36 [1.25-6.04]	2.13 [0.92, 4.91]	0.08	1.95 [0.34, 3.55]	0
Atopic asthmatics	21.8 (72)	2.65 [1.25-4.89]	**1.95 [1.05, 3.67]**	**0.04**	13.9 (61)	2.99 [1.25-4.48]	**3.23 [1.78, 5.87]**	**<0.01**	**2.34 [1.19, 3.49]**	0

*** Adjusted for the storage duration of EBC sample, sex and age at time of EBC collection.**

† Associations with a P-value of less than 0.05 are bolded.

‡ A total of 9 participants in both MACS and TAHS were excluded due to missing survey data on sensitization status.

Abbreviations: **EBC**: Exhaled breath condensate; **NO_x**: Total nitric oxides; **MACS**: Melbourne Atopy Cohort Study; **TAHS**: Tasmanian Longitudinal Health Study; **IQR**: Inter-quartile range; **OR**: Odds Ratio; **CI**: Confidence Interval.

Table 3: Associations between current rhinitis, rhinitis phenotypes and levels of EBC NO_x.

	MACS (n=356)				TAHS (n=424)				Meta-analysis	
	% (n)	NO_x (µM) Median [IQR]	OR* [95%CI]	P-value†	% (n)	NO_x (µM) Median [IQR]	OR* [95%CI]	P-value†	Pooled Estimate [95%CI]	I^2 (%)
Current rhinitis										
NO	63.2 (225)	1.25 [1.25-4.41]	1.00	-	39.6 (168)	1.25 [1.25-4.49]	1.00	-	1.00	-
YES	36.8 (131)	1.25 [1.25-4.87]	1.09 [0.70, 1.71]	0.70	60.4 (256)	2.81 [1.25-4.67]	**1.60 [1.08, 2.36]**	**0.02**	1.31 [0.82, 1.80]	33
Rhinitis phenotypes (MACS: n‡ =347, TAHS: n‡ =417)										
Non-atopic non-rhinitis	28.8 (100)	1.25 [1.25-3.44]	1.00	-	23.0 (96)	1.25 [1.25-3.22]	1.00	-	1.00	-
Atopic non-rhinitis	34.9 (121)	1.25 [1.25-5.51]	1.63 [0.92, 2.88]	0.09	16.6 (69)	3.27 [1.25-6.05]	**3.15 [1.67, 5.93]**	**<0.01**	2.08 [0.72, 3.45]	38
Non-atopic rhinitis	4.3 (15)	1.25 [1.25-5.55]	0.95 [0.30, 3.01]	0.93	20.1 (84)	1.92 [1.25-3.97]	1.81 [0.99, 3.29]	0.05	1.45 [0.57, 2.33]	0
Atopic (allergic) rhinitis	32.0 (111)	1.86 [1.25-4.60]	1.45 [0.81, 2.60]	0.21	40.3 (168)	3.42 [1.25-6.52]	**3.37 [2.00, 5.68]**	**<0.01**	2.24 [0.39, 4.09]	70

*** Adjusted for the storage duration of EBC sample, sex and age at time of EBC collection.**
† Associations with a P-value of less than 0.05 are bolded.
‡ A total of 9 participants in the MACS and 7 participants TAHS were excluded due to missing survey data on sensitization status.

Abbreviations: **EBC**: Exhaled breath condensate; **NO_x**: Total nitric oxides; **MACS**: Melbourne Atopy Cohort Study; **TAHS**: Tasmanian Longitudinal Health Study; **IQR**: Inter-quartile range; **OR**: Odds Ratio; **CI**: Confidence Interval.

Table 4: Possible interactions related to EBC NO_x in the TAHS cohort.

	OR* [95%CI]	P-value ‡
Current atopy† (P-value of interaction =0.05)		
ICS (-)	**2.86 [1.83, 4.47]**	**<0.01**
ICS (+)	0.64 [0.21, 1.96]	0.43
Current atopy* (P-value of interaction =0.01)		
Males	**4.44 [2.33, 8.48]**	**<0.01**
Females	1.47 [0.84, 2.58]	0.18
Age (P-value of interaction =0.01)		
Atopy (-)	0.45 [0.19, 1.06]	0.07
Atopy (+)	1.80 [0.76, 4.26]	0.18

*** Multivariate adjusted model for the storage duration of EBC sample, sex, age at time of EBC collection, current asthma, current rhinitis, sensitization, evver smoked, and any use of ICS in the last 12 months.**
† Associations with a P-value of less than 0.05 are bolded.
‡ In the previous 12 months.

Abbreviations: **EBC**: Exhaled breath condensate; **NO_x**: Total nitric oxide products; **TAHS**: Tasmanian Longitudinal Health Study; **OR**: Odds Ratio; **CI**: Confidence Interval; **ICS**: Inhaled corticosteroid.

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