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Metabolomic differences in lung function metrics: evidence from two cohorts

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Abstract

Rationale—The biochemical mechanisms underlying lung function are incompletely understood.

Objectives—To identify and validate the plasma metabolome of lung function using two independent adult cohorts: discovery—the European Prospective Investigation into Cancer-Norfolk (EPIC-Norfolk, n=10 460) and validation—the VA Normative Aging Study (NAS) metabolomic cohort (n=437).

Methods—We ran linear regression models for 693 metabolites to identify associations with forced expiratory volume in one second (FEV_1) and the ratio of FEV_1 to forced vital capacity (FEV_1/FVC), in EPIC-Norfolk then validated significant findings in NAS. Significance in EPIC-Norfolk was denoted using an effective number of tests threshold of 95%; a metabolite was considered validated in NAS if the direction of effect was consistent and p<0.05.

Measurements and main results—Of 156 metabolites that associated with FEV_1 in EPIC-Norfolk after adjustment for age, sex, body mass index, height, smoking and asthma status, 34 (21.8%) validated in NAS, including several metabolites involved in oxidative stress. When restricting the discovery sample to men only, a similar percentage, 18 of 79 significant metabolites (22.8%) were validated. A smaller number of metabolites were validated for FEV_1/FVC, 6 of 65 (9.2%) when including all EPIC-Norfolk as the discovery population, and 2 of 34 (5.9%) when restricting to men. These metabolites were characterised by involvement in respiratory track secretants. Interestingly, no metabolites were validated for both FEV_1 and FEV_1/FVC.

Conclusions—The validation of metabolites associated with respiratory function can help to better understand mechanisms of lung health and may assist the development of biomarkers.

INTRODUCTION

Metabolomics, the systematic profiling of the small molecules in a biological system,[1] represents a powerful tool to increase the understanding of the mechanisms of respiratory health, providing a downstream 'snapshot' of the status of a biological system reflecting phenotype as well as upstream genetic and environmental influences. As such, it is ideally suited to examine alterations in biological pathways that accompany phenotypical changes.[2] Several studies have successfully used metabolomics to explore diseases including asthma and COPD,[3] [4] suggesting there are measurable and biologically informative alterations in the metabolome that reflect perturbations in the respiratory system. However, only a small number of studies have investigated the metabolome of forced expiratory volume in one second (FEV_1) or the ratio of FEV_1 to forced vital capacity (FEV_1/FVC).[5-10] These metrics, which can be considered as measures of airflow obstruction, represent two of the most important indicators of pulmonary function in adults.[11] More broadly, given the known relationship between pulmonary function with systemic inflammation and a range of clinical pathologies from diabetes to cardiovascular disease, FEV_1 and FEV_1/FVC can also inform on overall health status.[5] Improving our understanding of the mechanisms of FEV_1 and FEV_1/FVC is therefore paramount, and consequently, metabolomic profiling of spirometric lung function warrants further investigation.

In this study, we aimed to identify and validate metabolites associated with FEV_1 and FEV_1/FVC, in two independent cohorts: the European Prospective Investigation into Cancer–Norfolk (EPIC-Norfolk)[12] and the VA Normative Aging Study (NAS).[13]

METHODS

Study populations

Discovery population—EPIC-Norfolk is a prospective population-based cohort[12] which recruited 25 639 men and women aged 40–79 years residing in Norfolk, England,

between 1993 and 1997. Information was collected on lifestyle variables and dietary habits; anthropometrics and blood samples were taken; and spirometry was performed. The majority (~93%) of blood samples were non-fasted[14 15] (online supplemental methods).

Validation population—NAS is a longitudinal study of ageing based in Boston, USA, that recruited 2280 men aged 21–80 between 1961 and 1970.[13] Participants have routine physical examinations and laboratory tests every 3–5 years, including fasting blood collection and spirometry. A subset of men with plasma samples collected during follow-up that were suitable for metabolomic profiling were selected to create the MAS Metabolomic Cohort.[16] All samples in this cohort were collected between 2000 and 2008. Samples collected prior to 2000 were not suitable for metabolomic profiling due to their storge conditions.

Metabolomic profiling

Metabolomic profiling was conducted by independently in each population by Metabolon (Durham, North Carolina, USA) using four non-targeted liquid chromatography–mass spectroscopy (LC-MS), platforms.[17] Metabolites were identified by mass-to-charge ratio, retention time and through a comparison to a library of purified known standards. Metabolites were matched to standards using LC-MS peaks and quantified using area under the curve. Data were processed according to the established quality control pipeline for each cohort[15 16 18-23] (online supplemental methods). As the metabolomic profiling was conducted independently in both cohorts, a slightly different subset of metabolites was measured in each. For validation purposes, these analyses were restricted to only those metabolites that were present and passed quality control (QC) in both cohorts. These common metabolites were matched between cohorts according to the unique Metabolon COMP.ID identifier.

Statistical analysis

Analyses were conducted in EPIC-Norfolk, and then validation of significant findings was performed in NAS. Linear regression models for two outcome variables, FEV_1 (as measured in litres) and FEV_1/FVC (expressed as a proportion), were run for each metabolite with adjustment for sex, age, body mass index (BMI), height, smoking status (never and current former) and asthma status. As NAS was all men, sex-stratified models were also run in EPIC-Norfolk.

The 'number of effective tests approach' was employed to account for multiple testing.[24 25] This method performs principal components analysis (PCA) on the full dataset in order to reduce the dimensionality of the dataset by creating new uncorrelated variables (principal components) that successively maximize variance. It then determines the number of principal components (PCs) required to explain a given percentage of variance in the data (ie, the number of effective tests). The adjusted p value threshold is calculated as m, which denotes the nominal p value threshold of 0.05, and m denotes the number of effective tests. For these analyses, we set a threshold of 95% variance explained (ENT95%). In EPIC-Norfolk, 415 PCs were required to explain 95% of the variance in the data, corresponding to a p value threshold of 1.205×10^{-4}. In EPIC-Norfolk men, it was 410 PCs and $p < 1.220 \times 10^{-4}$,

and in women 407 PCs and $p < 1.229 \times 10^{-4}$. In the validation analyses, we considered a metabolite validated if the p value is <0.05 and the direction of effect was consistent.

We conducted several sensitivity analyses to further explore the robustness of our findings. These included stratification of the EPIC-Norfolk subjects by BMI category and smoking status, additional adjustment for fasting status and prevalent diabetes and the inclusion of participants with self-reported emphysema or bronchitis at blood draw.

All analyses were conducted using R V.3.6.0 and Stata V.14.2. Significant metabolites were further explored using the 'Pathway Analysis' functionality in MetaboAnalyst V.4.0.[26] Pathway analysis tests for overenrichment of a given set of metabolites within a curated list of KEGG (Kyoto Encyclopedia of Genes and Genomes) defined metabolomic pathways while also taking pathway topology into account, that is, the positional importance of a metabolite within a pathway, to provide an overall enrichment p value and pathway impact score. All 693 metabolites that could be measured in both EPIC-Norfolk and in NAS were input as the reference metabolome. The hypergeometric test was specified for the over-representation analysis and relative 'betweenness' centrality for the pathway topology analysis.

RESULTS

Study population

The mean age of the 10 460 EPIC-Norfolk participants eligible for these analyses was 59.73 years (SD=8.95 years), and >99% were of white British/European descent (online supplemental table E1). Eleven per cent reported current smoking at the time of blood sample collection. The majority of the population had normal lung function as defined by their spirometry measures. Mean FEV_1 and FEV_1/FVC were 2.52 L (SD=0.72 L), and 0.82 (SD=0.11), respectively. The correlation between FEV_1 and FEV_1/FVC was r=0.183 ($p < 2.2 \times 10^{-16}$). Within EPIC-Norfolk, men were slightly older than women and were more likely to have a higher FEV_1 and a lower FEV_1/FVC.

NAS (online supplemental table E2) was older than EPIC-Norfolk, with a mean age of 75.12 years (SD=6.66) among the 437 men eligible for the NAS metabolomic cohort, but was similarly predominantly white with few current smokers (4.3%). The mean FEV_1 was 2.50 L (SD=0.60 L), and FEV_1/FVC was 0.74 (SD=0.08), both of which were lower than in EPIC-Norfolk, indicating slightly worse lung function in this older population. The correlation between FEV_1 and FEV_1/FVC was r=0.518, $p < 2.2 \times 10^{-16}$.

After data QC and processing, 1002 and 858 metabolites were available in EPIC-Norfolk and NAS, respectively. Of these, 693 were common to both datasets and included in these analyses.

Forced expiratory volume in one second

A total of 156 (22.5%) of 693 metabolites were associated ($p < ENT95\%$) with FEV_1 in EPIC-Norfolk (figure 1). The largest percentages were lipids (n=36, 23.1%) and amino acids (n=36, 23.1%). Half (n=78, 50%) were positively associated with FEV_1. The top hits were

threonate, where a 1-unit increase in threonate levels results in an increase in of 0.049 L in FEV_1 ($\beta=0.049$, 95% CI 0.040 to 0.058, $p=3.57\times10^{-25}$), oxalate ($\beta=0.046$, 95% CI 0.036 to 0.055, $p=8.46\times10^{-22}$) and ergothioneine ($\beta=0.047$, 95% CI 0.038 to 0.056, $p=1.62\times10^{-24}$). The most significant finding for inverse associations was cysteine sulfinic acid where a 1-unit increase in cysteine sulfinic acid levels was associated with a drop of 0.046 L in FEV_1 volume ($\beta=-0.046$, 95% CI -0.056 to -0.037, $p=3.16\times10^{-21}$). Pathway analyses determined the 156 metabolites were enriched for metabolic pathways including glycine, serine and threonine metabolism (p for enrichment$=3.33\times10^{-5}$), aminoacyl-tRNA biosynthesis (p for enrichment$=5.52\times10^{-4}$), caffeine metabolism (p for enrichment=0.007), and cysteine and methionine metabolism (p for enrichment=0.010) (online supplemental table E3).

In sex-stratified analyses, 79 (11.4%) and 74 (10.7%) metabolites were associated with FEV_1 in men and women, respectively. Forty were significant in both sexes (online supplemental table E4 and figure E1). Four metabolites were significant in men but not women, three of which were inversely associated with FEV_1: 1-stearoyl-2-arachidonoyl-Glycerophosphoethanolamine (18:0/20:4) and 1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4) (both phosphatidylethanolamines), and gamma-tocopherol/beta-tocopherol. One, 5alpha-pregnan-3beta,20alpha-diol monosulfate, was positively associated with FEV_1 in litres. Four metabolites, cysteine, 3-methoxycatechol sulfate and two metabolites of unknown identify were significant only in women, all were inversely associated with FEV_1. However, it should be noted, for both the male-only and female-only metabolites, the direction of effect was consistent in the other sex.

Validation in the NAS

Thirty-four (21.8%) of the 156 metabolites significant in the total EPIC-Norfolk sample were validated in the NAS (table 1), including several top hits, such as ergothioneine and beta-cryptoxanthine (online supplemental figure E2). It should be noted that due to differences in the metabolite normalisation and scaling procedures implemented prior to analysis, the scales of the beta-coefficients differed between the two studies.

Given NAS is all-male, we then explored the 79 metabolites significant in the EPIC-Norfolk men. Of these, 18 validated (22.8%). This was only a slightly greater level of validation than observed for the total population and a majority of these 18 (n=10, 55.6%) were also significant in EPIC-Norfolk women, suggesting little difference in the metabolome of FEV_1 by sex.

Ratio of FEV_1 to forced vital capacity

A total of 65 (9.4%) metabolites were significantly (p<ENT95%) associated with FEV_1/FVC in EPIC-Norfolk, of which the majority (n=52, 80.0%) were inversely associated with ratio (online supplemental table E5 and figure E3). The top hits included lactate, which was associated with a decrease in FEV_1/FVC proportion of 0.014 for every unit increase in lactate level ($\beta=-0.014$, 95% CI -0.016 to -0.012, $p=2.19\times10^{-4}$), 5-oxoproline ($\beta=-0.014$, 95% CI -0.016 to -0.011, $p=3.33\times10^{-38}$), sphingosine 1-phosphate ($\beta=-0.013$, 95% CI -0.015 to -0.011, $p=1.81\times10^{-37}$) and inosine ($\beta=0.011$, 95% CI 0.009 to 0.013, $p=7.09\times10^{-24}$). The 65 metabolites were enriched for metabolites of 12 pathways, including

alanine, aspartate and glutamate metabolism (p for enrichment=7.62×10^{-5}), arginine biosynthesis (p for enrichment=4.21×10^{-4}), the citrate cycle (p for enrichment=0.001), butanoate metabolism (p for enrichment=0.007) and aminoacyl tRNA biosynthesis (p for enrichment=0.009) (online supplemental table E3).

In sex-stratified analyses, 34 (4.9%) and 36 (5.2%) metabolites were significant in men and women, respectively. As with FEV_1, there was strong concordance between the sexes for FEV_1/FVC (online supplemental figure E4). Twenty-six metabolites were common to men and women: lactate, 5-oxoproline, sphingosine 1-phosphate and inosine were the most strongly associated in both sexes.

Validation in the NAS—Of the 65 metabolites significant in the total EPIC-Norfolk sample, 6 (9.2%) were validated in NAS (online supplemental figure E5): stearoylcarnitine (C18), oleoylcarnitine (C18:1), palmitoylcarnitine (C16), 5-methyluridine, cysteine s-sulfate and N-acetylglucosamine/N-acetylgalactosamine. All but 5-methyluridine were inversely associated with ratio (table 2).

Of the 34 significant in EPIC-Norfolk men, 2 (5.9%) were validated in NAS: oleoylcarnitine (C18:1) and N-acetylglucosamine/N-acetylgalactosamine.

Consistency of metabolite associations between FEV_1 and FEV_1/FVC

Figure 2 shows the unit changes for FEV_1 litres and FEV_1/FVC proportion with a 1-unit increase in metabolite level for every measured metabolite. When restricting to those that were validated in NAS, 34 metabolites were validated for FEV_1 and six for FEV_1/FVC. The two sets of metabolites were distinct, with no metabolite validated for both phenotypes. Figure 3 shows the direction of effect and p value from both EPIC-Norfolk and NAS for the 34 metabolites that were validated for FEV1 and the six validated for ratio. While for many metabolites the directions of effect were consistent for the two phenotypes and approached ENT95% significance, for others such as 1-palmitoyl-GPC (16:0) and 5-oxoproline, the directions of effect were discordant between phenotypes.

When considering the EPIC-Norfolk men as the discovery sample, 18 and 2 metabolites were validated for FEV_1 and FEV_1/FVC, respectively. Again, there were no common validated metabolites between the two phenotypes (online supplemental figure E6).

Sensitivity analyses

Individuals with self-reported emphysema or bronchitis at baseline were initially excluded from analyses. When running sensitivity analyses including the 1050 individuals in EPIC-Norfolk with these diagnoses, there was little change in the results. For both FEV_1 and FEV_1/FVC, the vast majority of significant metabolites were robust to the inclusion of the new subjects, including 100% of the validated metabolites. There were also a small number of novel metabolites that were validated when including the emphysema and bronchitis cases (online supplemental table E6).

Additional adjustment for fasting status and diabetes status in EPIC-Norfolk also had little influence on our findings. In the fasting adjusted models, 154 of the 156 (98.7%)

metabolites were associated with FEV_1 in the primary analysis retained significance with adjustment for fasting status, including all 34 of the metabolites that could be validated in NAS. For FEV_1/FVC, 64 of 65 retained significance with adjustment for fasting status, including all 6 of the validated metabolites (results not shown). There were 371 cases of prevalent diabetes in the EPIC-Norfolk dataset. When diabetes status was adjusted for, 154 of the 156 (98.7%) metabolites associated with FEV_1 in the primary analysis retained significance with adjustment for fasting status. All but 1 of the 34 validated metabolites, (1-(1-enyl-palmitoyl)-GPC (P-16:0)), retained significance, and it should be noted this metabolite almost reached ENT95% significance. For FEV_1/FVC, again 64 of 65 retained significance with adjustment for fasting status, including all 6 of the validated metabolites (results not shown).

We further ran our EPIC-Norfolk models stratified by WHO-defined BMI category, for healthy (n=4189), overweight (n=4750) and obese (n=1477) participants. We note that the number of individuals in the underweight category (n=44) was too small to run analyses. Seventy (10.1%), fifty-four (7.8%) and four (0.6%) metabolites were associated with FEV_1 at ENT95% in healthy, overweight and obese individuals, respectively (online supplemental table E7). Of these, 28 were common between the healthy and overweight individuals. Six of the 28 common metabolites were also among those validated in NAS; these included a number of the top hits from the primary analysis: ergothioneine, beta-cryptoxanthin, X-12117, N2,N2-dimethylguanosine, 2-aminobutyrate and N1-methyladenosine.

Twenty-nine (4.2%), 28 (4.0%) and 10 (1.4%) metabolites were associated with FEV_1/FVC at ENT95% in healthy, overweight and obese participants, respectively (online supplemental table E8). Of these, seven were significant in all three groups. The top validated hit for FEV_1/FVC in the primary analysis, oleoylcarnitine (C18:1), was significant in the both healthy and overweight individuals. Even where ENT95% significance was not reach for both FEV_1 and FEV_1/FVC, the direction of effect was consistent and at least $p<0.05$ was reached for most metabolites across all the groups.

Finally, we ran our EPIC-Norfolk models stratifying by smoking status at blood draw: current (n=4867), former (n=4750) and never (n=1145). Fifty-eight (8.4%), 65 (9.4%) and 20 (2.9%) metabolites were associated with FEV_1 at ENT95% in never, former and current smokers, respectively. Never and former smokers were more alike in their associations than current smokers. However, three metabolites were ENT95% significant in all groups and were among the validated hits: ergothioneine, beta-cryptoxanthin and N1-methyladenosine (online supplemental table E9). For FEV_1/FVC, 32 (4.6%), 30 (4.3%) and 2 (0.3%) metabolites were significant at ENT95% in never, former and current smokers, respectively. The top hit, oleoylcarnitine (C18:1), was ENT95% significant in never and former smokers (online supplemental table E10). Again, even where ENT95% significance was not reached for both FEV_1 and FEV_1/FVC, the direction of effect was consistent and at least $p<0.05$ was reached for most metabolites across all the groups.

DISCUSSION

Pulmonary function, as measured by FEV_1 and FEV_1/FVC, reflects the physiological state of the lung.[5] Common chronic lung diseases such as asthma, COPD, emphysema, chronic bronchitis and pulmonary fibrosis are characterised by impairments in pulmonary function. Measures of lung function as assessed by spirometry can be used for diagnosis, assessment of severity and monitoring.[11] Furthermore, there is evidence that lung function and its associated diseases have far-reaching systemic effects beyond the lung, including on the cardiovascular, nervous and immune systems.[27] Poor lung function has been linked to chronic inflammation, as well as the development of other chronic conditions including diabetes, kidney and cardiovascular disease.[5 28 29] What's more, reduced FEV_1 has been shown to be correlated with higher mortality, irrespective of disease and smoking status.[30] As such, elucidating the underlying biological mechanisms of lung function is crucial to improve our understanding not only of the lung, but of the pathogenesis of multiple diseases and morbidity.

Individuals' pulmonary function is a product of the interactions between their underlying genetics and their environmental exposures.[31-33] Consequently, it is ideally suited to metabolomics profiling, which reflects the downstream products of these interactions across the life course.[2]

In this study, we identified and validated metabolites measured in the circulating blood that were associated with lung function. One hundred and fifty-six metabolites were significantly associated with FEV_1 with stringent correction for multiple testing in over 10 000 subjects from EPIC-Norfolk, a large population-based cohort of men and women residing in the UK. Of these, 34 (21.8%) could be validated in 437 men from the US based NAS. A slightly higher proportion, 22.8%, validated when restricting the EPIC-Norfolk discovery sample to men only. For FEV_1/FVC, a much smaller number of metabolites were found to be significant in the total EPIC-Norfolk population, 65 (9.4%) of which 6 were validated. Only 2 of 34 were validated when restricting to EPIC-Norfolk men. Interestingly, there were no metabolites that were validated for both FEV_1 and FEV_1/FVC. This is in agreement with existing metabolomics studies of lung function[5] and corresponds to other omic studies which report a smaller number of genetic associations for FEV_1/FVC compared with FEV_1.[33] Nevertheless, validated metabolites were found for both metrics that inform on underlying biology. Given the unique position of the metabolome closest to phenotype and the relative ease and cost-effectiveness of measuring the metabolome in blood, metabolites that reflect a physiological state may have translational utility in biomarker development.

Few studies have explored the global metabolome of FEV_1 or FEV_1/FVC. In a multiethnic study of lung function, Yu *et al* identified 95 serum metabolites associated with FEV_1 that were characterised by branched chain amino acids and glutamine.[5] As in this current analysis, they identified fewer FEV_1/FVC-associated metabolites, and these were primarily lipids. Two studies by Menni *et al* within the TWINS UK study also reported several associations between FEV_1 and serum levels of amino acids, particularly of the glycine, serine and threonine metabolism pathway,[6 10] while other studies in blood report strong correlations between FEV_1 with kynurenine, several sphingomyelins and sphingolipids.[9 34]

In a cohort of patients with asthma–COPD overlap, urinary histidine was negatively correlated with both FEV_1 and FEV_1/FVC.[7] Finally, Halper-Stromberg *et al* explored plasma and bronchoalveolar lavage (BAL) fluid, observing there was little correlation between the results for the two biosamples. The greatest number of associations were found for BAL, but that both BAL and plasma provided distinct biologically important information.

Despite differences between these studies and ours, there was high consistency with our findings. The strongest association with FEV_1 in the EPIC-Norfolk total population was with thre-onante, a major metabolite of the antioxidant ascorbic acid,[10] higher levels of which were associated with increased FEV_1, indicating a better oxidative state. Although threonante was not validated in the NAS, it has previously been associated with FEV_1 in other studies.[5 10] Our results were also consistent with existing literature for many of our validated hits. Metabolites of glycine, serine and threonine metabolism, demonstrated some of the strongest associations with FEV_1 in our analyses and have been implicated in lung function,[5 7 10] as well as in COPD[35] and asthma,[4] due to their role in oxidative stress. Oxidative stress is a product of increased exposure to free radicals and/or decreased antioxidant defence mechanisms, and given its proximity to ambient air, the respiratory system is particularly susceptible. Exposure to reactive oxidant species causes mitochondrial DNA damage and disruption of the electron transport chain, leading to overall reduced pulmonary function.[36]

In common with previous literature,[5] we also observed an enrichment of metabolites of the aminoacyl-tRNA biosynthesis pathway, a pathway that is similarly involved in oxidative stress and which plays a central role in protein biosynthesis by catalysing the attachment of an amino acid to the 3′ end of its cognate tRNA.[37] The susceptibility of the lungs to oxidative stress[38] may also explain our validated association with beta-cryptoxanthin, an oxygenated carotenoid that acts as an antioxidant.[39] Dietary supplementation of beta-cryptoxanthin has been shown to improve pulmonary function,[40] and our findings may provide mechanistic support role the role of beta-crytoxanthin as a supplement. It is also worth noting that the association between this metabolite and FEV_1 was observed across all smoking categories and in both healthy-weight and overweight individuals, strengthening its potential as therapeutic strategy. Our validated hits for FEV_1 also included other metabolites previously reported in the literature,[5] for example, two nucelosides, N2, N2-dimethylguanosine and pseudouridine, and the carbohydrate mannose.

We further validated several novel associations with FEV_1, the majority of which involve metabolites previously associated with COPD, asthma or oxidative stress. Our top validated hit was the xenobiotic ergothionine, a sulfur-containing derivative of histidine, suggested to have antioxidant properties,[41] which was also robust to stratification by smoking status and BMI category. We validated 4-allylphenol sulfate, which like ergothionine is a xenobiotic originating from food and is positively associated with FEV_1. Interestingly, levels of these two metabolites were increased with a decrease in sodium intake in the Dietary Approaches to Stop Hypertension trial,[42] and lower sodium intake has been associated with improved lung function.[43] This again suggests that supplementation with these metabolites may be

beneficial for the improvement of FEV_1. Clinical trials are necessary to explore this question further.

Among the six metabolites that validated for FEV_1/FVC were three acylcarnitines, which were associated with a lower ratio and, therefore, greater airway obstruction. Accumulation of acyl carnitines in the pulmonary surfactant of the lung has previously been shown to sensitise lungs to injury by inhibiting the surface adsorption of pulmonary surfactant and its ability to reduce surface tension.[44] Levels of acylcarnitines have also been shown to be higher in the plasma of individuals with pulmonary hypertension, who tend to have a lower FEV_1/FVC.[45] Further, our top hit, oleoylcarnitine (C18:1), was also identified by Yu *et al* as associated with both FEV_1 and $FEV_1:FVC$[5] and was robust to stratification by smoking status and BMI in these analyses. Interestingly, we also validated N-acetylglucosamine/N-acetylgalactosamine, a glycoprotein which forms an important component of airway mucins, another respiratory tract secretant.[46] Finally, we validated a positive association between 5-methyluridine (ribothy-midine) and FEV_1/FVC, in agreement with our previous work demonstrating that higher levels of ribothymidine can attenuate the decline in bronchodilator response that occurs with age.[47]

There were a number of limitations to these analyses. There was a large discrepancy in power between our discovery and validation populations, and it is possible that those metabolites which we were able to validate in the smaller NAS cohort represent those with the largest effect estimates. It is likely other associations in EPIC-Norfolk represent true positives that the NAS cohort was too underpowered to detect. Validation was likely also hindered by the differences in the underlying populations of the two cohorts. Our discovery population was younger, and in addition to the known influence of age on the metabolome,[48] lung function also changes as an individual ages due to physiological changes in lung elasticity and muscle tone and decreased surface area for alveolar gas exchange, in addition to immunological changes.[49] As such, the metabolomic changes associated with FEV_1 and FEV_1/FVC may also differ by age, again decreasing the potential for replication between the cohorts.

EPIC-Norfolk included both men and women, while our validation population was all-male, and sex is also known to influence the metabolome and lung function.[50 51] Nevertheless, there was high concordance in the significantly associated metabolites by sex for both FEV_1 and FEV_1/FVC in EPIC-Norfolk. Even when significance in one sex was not met in the other, the directions of effect were consistent. Of the 39 metabolites that were significant in men but not women for FEV_1, 31 were nominally significant ($p<0.05$). Similarly, for FEV_1/FVC, seven of the eight 'male-only' metabolites were nominally significant in women. When attempting to validate our findings in the NAS, there was a similar level of validation when using the metabolites identified in the EPIC-Norfolk total and all-male populations. Taken together, these results suggest little difference in metabolomics of lung function by sex. Although our cohort was almost entirely Caucasian, and for this reason, to maintain stability of our models, we did not adjust for race; results from the previous multiethnic studies[5] and the consistency of our findings with these studies suggest the metabolome of lung function also does not differ greatly by race.

Our results were also largely consistent, particularly with regard to the validated metabolites across healthy and overweight individuals and former and current smokers. There were some differences in the metabolite –lung function associations in the individuals who were obese and those who were current smokers. There is a biological rationale as to why these relationships may differ; both BMI and smoking have metabolomic consequences[52][53]; and smoking has a well-characterised determinantal effect on spirometrics.[54] The association with obesity was less well characterised; although it reduces lung function overall due to both mechanical effects on the chest wall and immunological changes, FEV_1 and FEV_1/FVC have been reported to be only mildly affected except in cases of extreme obesity (62 kg/m^2).[55] Obese subjects and current smokers were also our smallest groups and had reduced power; therefore, further work is needed to disentangle these findings.

In both populations, the majority of participants had good to moderate lung function, potentially limiting the generalisability of our findings to populations with very poor lung function. However, sensitivity analyses including individuals with emphysema and bronchitis did not manifestly affect our results and had little influence on our overall conclusions. We did validate a small number of additional metabolites with the additional participants, providing further support for the roles of glutamate metabolism and kynurenine in lung function.[9][56]

These analyses were based on two existing datasets that were generated independently and have been published on previously.[15][16][18-21] The spirometry measures in EPIC-Norfolk were not specifically defined according to American Thoracic Society or European Respiratory Society standards. However, we note that the spirometer used in EPIC Norfolk has demonstrated good sensitivity and specificity for identifying COPD compared with the gold standard, and the EPIC-Norfolk spirometry measures have previously been published in peer-reviewed publications.[57][58] As we used pre-existing blood samples for these analyses collected prior to widespread use of untargeted metabolomic profiling, neither of the cohorts' blood collection procedures were optimised to metabolomics. Time of day of collection varied across the cohorts and diurnal variation in the metabolome may also have influenced our findings. We note that, to date, there are no gold standards in the QC or data processing of metabolomic data.[59] As such, there were some differences in the QC pipelines of the two datasets, and this may have hampered validation of a larger number of metabolites. Samples in Epic-Norfolk were between 17 and 24 years old at profiling, and the NAS samples were between 10 and 18 years old. It has been shown that long-term storage can influence the levels of certain metabolites,[60] which may have affected our results. Our discovery and replication approach, including samples with a range of storage times, limits the potential for false-positive findings; however, we cannot rule out the possibility we may have missed metabolites of interest due to the age of our samples.

We did not see any overlap in terms of specific metabolites associated with FEV_1/FVC between our plasma study and Halper-Stromberg's BAL analysis.[8] However, there are a number of disadvantages to using BAL including its cost, discomfort to the patient, difficulty of obtaining the sample and the related risk of contamination.[61] While blood may not be the biofluid closest to the lung, given lung function has shown to be reflected systemically,[5][27-29] blood can still inform on the processes occurring there. As such,

recognising both BAL samples and blood provide vital insights into the biochemical mechanisms of lung function, we found that blood, which is simple and cost-effective to collect, may be superior for the development of future clinically translatable biomarkers of lung function.

This study also had several strengths: it represents the largest study to date to focus on the metabolome of FEV_1 and FEV_1/FVC, and the first to consider differences by sex. Crucially, we were able to validate our findings in an independent sample, profiled using the same platform as the discovery population.[17] The differences between our two cohorts would only have biased our results to the null, and we note that additional adjustment for fasting status in EPIC-Norfolk did not alter our results or conclusions. Additionally, our results were robust to additional adjustment for prevalent diabetes status, a common comorbidity in populations in the age range of those included in these analyses. We validated multiple biologically relevant metabolites, confirming findings from prior literature and identifying novel ones. We used a stringent correction for multiple testing to obtain the most robust results possible. At a less stringent discovery p value in EPIC-Norfolk, there would have been even greater validation in the NAS. Therefore, additional validation of these metabolites in other populations may be warranted. Given the cross-sectional nature of this study, we cannot determine whether the observed metabolomic perturbations are the cause of reduced lung function or an effect. Therefore, follow-up studies should consider repeated longitudinal sampling, as well as absolute quantification to determine their biomarker potential. We included unnamed metabolites in our analysis to reflect the fact that Metabolon is continually updating its library and may in the future be able to assign names to these metabolites.

In conclusion, this study is among the first to validate metabolites significantly associated with lung function parameters. These findings indicate that oxidative stress is correlated with an individual's FEV_1, while the composition of respiratory secretants seems to be of importance for FEV_1/FVC. It is of note that two dietary compounds were strongly associated with FEV_1, indicating the potential of simple dietary interventions to improve lung health. We can therefore use these results to both better understand the mechanisms of lung function and support the development of future biologically informative biomarkers or therapeutic strategies for the overall improvement of pulmonary care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Data availability statement

Data are available upon reasonable request. Data are available on request with agreement from all coauthors and funding bodies.

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What is the key question?

- To determine the, as yet not fully understood, systemic effects of lung function.

What is the bottom line?

- This is the largest study to identify validated metabolomic associations with forced expiratory volume in one second (FEV_1) and ratio of FEV_1 to forced vital capacity, providing novel insights into the mechanisms underlying lung function.

Why read on?

- These findings pave the way for the development of novel biomarkers or therapeutic strategies for improved lung function.
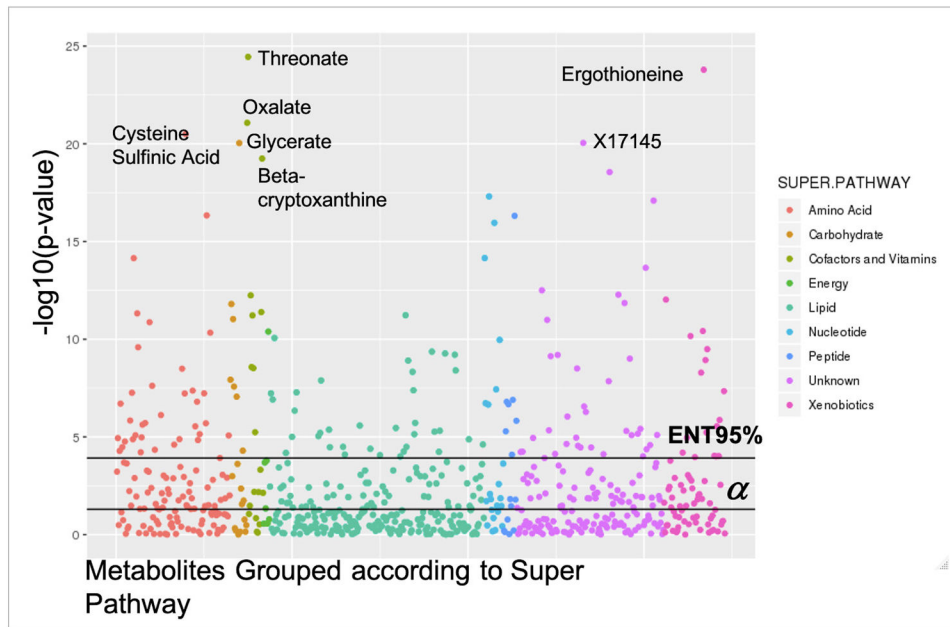
Figure 1.
Manhattan plot demonstrating the strength of association between FEV_1 and 693 metabolites in European Prospective Investigation into Cancer–Norfolk, coloured according to Metabolon Superpathway. Top metabolite hits are identified according to their common name. Metabolites with the format Xnnnnn are of unknown identity but can be tracked and quantified, and therefore Metabolon is confident they represent biologically relevant molecules and not analytical artefacts. The black horizontal lines indicate significance thresholds. FEV_1, forced expiratory volume in one second.
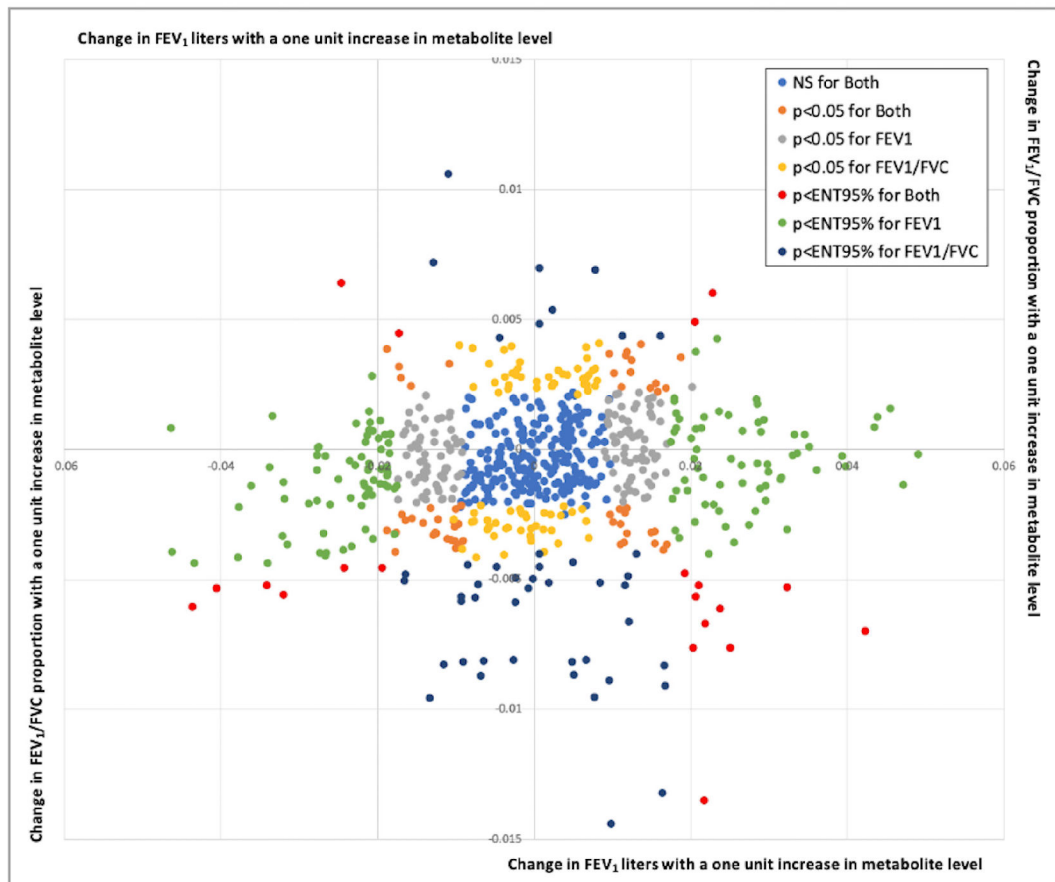
Figure 2.
Effect estimates of metabolite association with FEV_1 and FEV_1/FVC in European Prospective Investigation into Cancer–Norfolk, coloured according to significance of effect. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; NS, not significant.

Figure 3.
Direction of effect and p value in the validated lung function-associated metabolites for (A) EPIC-Norfolk men and NAS for 34 FEV_1-validated metabolites, (B) EPIC-Norfolk men and NAS for 6 FEV_1-validated metabolites. To allow comparison between FEV_1 and FEV_1/FVC, the effect estimates for both are given for each set of metabolites in each cohort. ° indicates a metabolite has not been confirmed using an analytical standard, but Metabolon is confident in its identity based on its analytical parameters. Metabolites with the format Xnnnnn are of unknown identity but can be tracked and quantified, and therefore, Metabolon is confident they represent biologically relevant molecules and not analytical artefacts. EPIC, European Prospective Investigation into Cancer; EPIC-Norfolk, European Prospective Investigation into Cancer–Norfolk; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; NAS, Normative Aging Study.

Table 1

FEV$_1$-associated metabolites validated in the VA NAS

Metabolite	Super pathway	EPIC-Norfolk total		EPIC-Norfolk men		VA NAS	
		Change in FEV$_1$ litres with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV$_1$ litres with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV$_1$ litres with a 1-unit increase in metabolite level β (95% CI)	P value
2-aminobutyrate *	Amino acid	0.04 (0.03 to 0.04)	7.12E-15	0.03 (0.02 to 0.05)	1.59E-05	0.17 (0.03 to 0.31)	1.81E-02
Imidazole propionate *	Amino acid	−0.03 (−0.04 to −0.02)	1.35E-11	−0.05 (−0.06 to −0.03)	1.62E-08	−0.08 (−0.15 to −0.01)	3.04E-02
Vanillylmandelate *	Amino acid	−0.03 (−0.04 to −0.02)	4.63E-11	−0.03 (−0.05 to −0.02)	5.64E-05	−0.21 (−0.32 to −0.1)	1.97E-04
N-formylmethionine *	Amino acid	−0.03 (−0.04 to −0.02)	5.97E-08	−0.03 (−0.04 to −0.01)	1.64E-03	−0.23 (−0.38 to −0.08)	3.73E-03
N-acetylputrescine *	Amino acid	−0.02 (−0.03 to −0.02)	1.58E-07	−0.03 (−0.05 to −0.02)	5.97E-05	−0.24 (−0.37 to −0.1)	5.34E-04
5-oxoproline	Amino acid	0.02 (0.01 to 0.03)	1.45E-06	0.02 (0.01 to 0.03)	5.77E-03	0.14 (0.06 to 0.23)	1.10E-03
5-methylthioadenosine	Amino acid	−0.02 (−0.03 to −0.01)	2.88E-06	−0.03 (−0.04 to −0.01)	7.92E-04	−0.12 (−0.21 to −0.04)	5.66E-03
Kynurenine	Amino acid	−0.02 (−0.03 to −0.01)	7.20E-06	−0.03 (−0.05 to −0.01)	4.93E-04	−0.19 (−0.34 to −0.04)	1.21E-02
Threonine	Amino acid	0.02 (0.01 to 0.03)	8.20E-06	0.03 (0.02 to 0.05)	8.02E-05	0.21 (0.05 to 0.37)	1.10E-02
N-acetylalanine	Amino acid	−0.02 (−0.03 to −0.01)	1.15E-05	−0.02 (−0.04 to −0.004)	1.27E-02	−0.21 (−0.39 to −0.04)	1.57E-02
N-acetylserine *	Amino acid	−0.02 (−0.03 to −0.01)	6.11E-05	−0.02 (−0.03 to −0.001)	5.77E-02	−0.22 (−0.37 to −0.08)	2.53E-03
Mannose	Carbohydrate	−0.02 (−0.03 to −0.02)	8.73E-08	−0.03 (−0.04 to −0.01)	1.36E-04	−0.1 (−0.16 to −0.03)	3.53E-03
Beta-cryptoxanthin *	Cofactors and vitamins	0.04 (0.03 to 0.05)	5.68E-20	0.05 (0.03 to 0.06)	4.27E-09	0.14 (0.06 to 0.21)	2.36E-04
1-(1-enyl-palmitoyl)-2-linoleoyl-GPC(P-16:0/18:2) *†	Lipid	0.03 (0.02 to 0.04)	4.31E-10	0.05 (0.03 to 0.07)	2.94E-10	0.2 (0.07 to 0.33)	2.93E-03
Glycochenodeoxycholate sulfate	Lipid	−0.03 (−0.04 to −0.02)	6.24E-10	−0.04 (−0.06 to −0.03)	1.30E-07	−0.09 (−0.16 to −0.02)	1.56E-02
Linoleoyl ethanolamide	Lipid	0.02 (0.01 to 0.03)	4.57E-07	0.03 (0.02 to 0.05)	4.30E-05	0.12 (0.04 to 0.2)	4.69E-03
1-palmitoyl-GPC (16:0)	Lipid	0.02 (0.01 to 0.03)	2.63E-06	0.02 (0.01 to 0.04)	4.84E-03	0.18 (0.02 to 0.35)	2.88E-02
Linoleoylcarnitine (C18:2) †	Lipid	0.02 (0.01 to 0.03)	9.07E-06	0.03 (0.02 to 0.05)	1.26E-05	0.11 (0.02 to 0.2)	2.35E-02
1-(1-enyl-palmitoyl)-GPC (P-16:0) *†	Lipid	0.02 (0.01 to 0.03)	8.63E-05	0.02 (0.01 to 0.04)	1.92E-03	0.17 (0.04 to 0.3)	1.05E-02
N2,N2-dimethylguanosine *	Nucleotide	−0.04 (−0.05 to −0.03)	4.92E-18	−0.04 (−0.06 to −0.03)	2.72E-07	−0.29 (−0.43 to −0.15)	6.58E-05
N1-methyladenosine *	Nucleotide	−0.04 (−0.05 to −0.03)	7.05E-15	−0.04 (−0.06 to −0.02)	2.54E-06	−0.18 (−0.34 to −0.02)	2.47E-02
Pseudouridine *	Nucleotide	−0.03 (−0.04 to −0.02)	1.08E-10	−0.04 (−0.05 to −0.02)	3.86E-05	−0.22 (−0.37 to −0.07)	3.55E-03

Metabolite	Super pathway	EPIC-Norfolk total		EPIC-Norfolk men		VA NAS	
		Change in FEV_1 litres with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV_1 litres with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV_1 litres with a 1-unit increase in metabolite level β (95% CI)	P value
N6-carbamoylthreonyl adenosine [*]	Nucleotide	−0.03 (−0.04 to −0.02)	1.89E-07	−0.02 (−0.04 to −0.004)	1.58E-02	−0.16 (−0.26 to −0.06)	2.21E-03
7-methylguanine [*]	Nucleotide	−0.02 (−0.03 to −0.02)	2.20E-07	−0.03 (−0.04 to −0.01)	1.14E-03	−0.19 (−0.36 to −0.02)	2.63E-02
X-12117 [*]	**Unknown**	**−0.05 (−0.06 to −0.04)**	**2.81E-19**	**−0.06 (−0.08 to −0.05)**	**8.22E-13**	**−0.13 (−0.2 to −0.05)**	**7.32E-04**
X-11315 [*]	**Unknown**	**0.03 (0.03 to 0.04)**	**2.21E-14**	**0.03 (0.02 to 0.05)**	**2.89E-05**	**0.14 (0.04 to 0.23)**	**5.87E-03**
X-18901	**Unknown**	**0.02 (0.01 to 0.03)**	**2.76E-07**	**0.04 (0.02 to 0.05)**	**8.54E-07**	**0.09 (0.01 to 0.17)**	**3.53E-02**
X-23590 [*]	Unknown	−0.02 (−0.03 to −0.01)	3.87E-06	−0.02 (−0.03 to −0.003)	2.26E-02	−0.11 (−0.2 to −0.02)	2.02E-02
X-16087 [*]	Unknown	−0.02 (−0.03 to −0.01)	4.95E-06	−0.02 (−0.04 to −0.01)	7.39E-03	−0.13 (−0.21 to −0.06)	7.52E-04
X-15 728	Unknown	0.02 (0.01 to 0.03)	2.85E-05	0.03 (0.01 to 0.05)	6.33E-04	0.09 (0.04 to 0.14)	7.37E-04
X-15 486	Unknown	−0.02 (−0.03 to −0.01)	5.71E-05	−0.02 (−0.04 to −0.01)	4.93E-03	−0.14 (−0.23 to −0.05)	2.07E-03
X-21 258	Unknown	0.02 (0.01 to 0.03)	5.90E-05	0.03 (0.01 to 0.04)	4.09E-04	0.07 (0.001 to 0.13)	4.88E-02
4-allylphenol sulfate	**Xenobiotics**	**0.03 (0.02 to 0.04)**	**3.81E-11**	**0.04 (0.02 to 0.06)**	**2.76E-07**	**0.09 (0.01 to 0.16)**	**2.72E-02**
Ergothioneine [*]	**Xenobiotics**	**0.05 (0.04 to 0.06)**	**1.62E-24**	**0.06 (0.04 to 0.07)**	**9.80E-14**	**0.1 (0.01 to 0.19)**	**2.43E-02**

18 metabolites in bold are those that are also significant at an ENT95% threshold in EPIC-Norfolk men and validated in NAS.

Metabolites with the format Xmmmm are of unknown identity but can be tracked and quantified, and therefore, Metabolon is confident they represent biologically relevant molecules and not analytical artefacts.

[*]
ENT95% significant in EPIC-Norfolk women.

[†]Indicates a metabolite has not been confirmed using an analytical standard, but Metabolon is confident in its identity based on its analytical parameters.

EPIC-Norfolk, European Prospective Investigation into Cancer–Norfolk; FEV_1, forced expiratory volume in one second; NAS, Normative Aging Study.

Table 2

FEV$_1$:FVC-associated metabolites validated in the NAS

Metabolite	Super pathway	EPIC-Norfolk total		EPIC-Norfolk men		VA NAS	
		Change in FEV$_1$/FVC with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV$_1$/FVC with a 1-unit increase in metabolite level β (95% CI)	P value	Change in FEV$_1$/FVC with a 1-unit increase in metabolite level β (95% CI)	P value
Cysteine s-sulfate	Amino acid	−0.005 (−0.007 to −0.003)	1.70E-05	−0.004 (−0.008 to 0.001)	1.88E-02	−0.011 (−0.02 to −0.001)	3.03E-02
N-acetylglucosamine/N-acetylgalactosamine	**Carbohydrate**	**−0.006 (−0.008 to −0.003)**	**3.02E-07**	**−0.007 (−0.01 to −0.003)**	**8.92E-05**	**−0.022 (−0.042 to −0.001)**	**3.98E-02**
Oleoylcarnitine (C18:1) *	**Lipid**	**−0.009 (−0.011 to −0.007)**	**1.83E-16**	**−0.01 (−0.013 to 0.007)**	**2.86E-09**	**−0.016 (−0.031 to 0.001)**	**3.15E-02**
Stearoylcarnitine (C18)	Lipid	−0.005 (−0.007 to −0.003)	1.88E-06	−0.006 (−0.01 to −0.003)	1.98E-04	−0.017 (−0.034 to −0.001)	4.27E-02
Palmitoylcarnitine (C16)	Lipid	−0.005 (−0.007 to −0.002)	2.55E-05	−0.005 (−0.008 to 0.002)	2.08E-03	−0.019 (−0.037 to −0.002)	2.96E-02
5-methyluridine (ribothymidine)	Nucleotide	0.004 (0.002 to 0.006)	4.97E-05	0.005 (0.002 to 0.009)	2.63E-03	0.023 (0.001 to 0.046)	4.15E-02

2 Metabolites in bold are those that are also significant at an ENT95% threshold in EPIC-Norfolk men and validated in NAS.

*
ENT95% significant in EPIC-Norfolk women.

EPIC-Norfolk, European Prospective Investigation into Cancer–Norfolk; FEV$_1$/FVC, ratio of FEV$_1$ to forced vital capacity; NAS, Normative Aging Study.