Non-invasive Phenotyping using Exhaled Volatile Organic Compounds in Asthma

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ONLINE DATA SUPPLEMENT

Methods

Inclusion and exclusion criteria

Inclusion criteria for the asthmatic subjects were as follows: physician diagnosis of asthma with current asthma symptoms, treatment with at least a short-acting inhaled β-agonist, age $\geq$ 18 years. Subjects were excluded if they were current smokers, or on continuous oral corticosteroids, Subjects with a clinical history of asthma exacerbation within 4 weeks, defined as worsening of asthma symptoms requiring a change in therapy by a physician or a change in regular asthma therapy (including short course of oral corticosteroids), were excluded. Healthy volunteers were never-smokers without respiratory symptoms, and with a baseline FEV_1 of greater than 85% predicted and FEV_1/FVC greater than 0.7.

Breath sample analysis by gas chromatography mass spectrometry

Samples were analysed in random order. Adsorbent tubes underwent two-stage thermal desorption (Markes Unity desorption unit, Markers International, Llantrisant, UK) by first heating the adsorbent tube to 250°C for 7 min and directing them at 50 ml / min towards a cold trap kept at 1°C. At the second stage the cold trap was heated for 10 min at 280°C and volatiles sent to the gas chromatography (GC) column for separation, then analysed by time of flight mass spectrometry (ToF-MS, GCT Premier, Waters Corp., Manchester, UK). An Agilent 6890 oven was equipped with a (5%-phenyl)-methylpolysiloxane column (30m, 0.25mm internal diameter, 0.1µm film thickness, Agilent Technologies, West Lothian, UK). The GC oven operated on a ramp program with an initial temperature of 40°C, ramp to 170°C at 6°C/min, then ramp to 190°C at 15°C/min. A post-run was set up where the oven was ramped from 190°C to 250°C for 2

min. The ToF-MS (7000 Resolution) was in electron ionisation mode set up at 70eV, source temperature 200°C and spectra were recorded in dynamic range extension mode at 10 scan/s over a range of 50 – 650m/z.

Sputum induction and processing

Prior to sputum induction, baseline spirometry was conducted and subjects given 200 mcg salbutamol via spacer. Subjects then underwent 5 minute inhalations of hypertonic saline (3%, 4%, 5%) via ultrasonic nebulizer for a total of 15 minutes, Spirometry was conducted between inhalations and at the end of sputum induction for safety, and subjects given further salbutamol only if required. Sputum was stored on ice until processed. Sputum plugs were isolated from saliva component and weighed. Sputum was processed with 0.1% DTT, cell counts were performed and cytospins made. Cytospins were stained and a differential cell count conducted by two observers on 400 cells. Those with significant squamous contamination (> 20%) were deemed to be uncountable

Statistical analysis

All data were analysed using SPSS version 15 (SPSS Inc., Chicago, IL, USA). For demographic data, descriptive statistics were used, with between group comparisons made using Pearson chi square, parametric (students t-test) and non parametric tests (Mann Whitney U) where appropriate.

For each comparison of interest (e.g. asthma *versus* healthy controls; eosinophilia *versus* non-eosinophilia etc) initial dimension reduction was performed by repeated univariate analysis (logistic regression) of the identified compounds. Variables were retained based on a p value set at 0.10 to maximise the number of variables included

whilst ensuring the subsequent PCA was valid. The validity of PCA was determined using the Kaiser-Meyer-Olkin measure of sampling adequacy (minimum set at 0.5) which measures the ratio of sample size to number of compounds, and Bartlett's test of sphericity which measures the correlations between the compounds. Principal components (PCs) were extracted and retained based on Kaiser's criterion of eigenvalues ≥ 1, and the direct oblimin rotation was applied. The PCs were entered into multivariate logistic regression to generate a best-fit model for between group discrimination. Because we did not have any information on the relative importance of each variable both the backward and the forward stepwise method of regression was used. The performance of this model was described by receiver operating characteristics [1]. A second method, PC-discriminant function analysis (DFA) was used in parallel to check the validity of the model.

Reference

1. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 1993; 39: 561-577.