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Non-invasive sampling methods of inflammatory biomarkers in asthma and allergic rhinitis

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CHAPTER 6

Combining alternative sputum processing methods & sensitive detection techniques for biomarker analysis: a feasibility study

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Abstract

Rationale Traditional biomarker quantification methods in exhaled breath condensate (EBC) and sputum have yielded conflicting data due to the lack of standardization of the collection technique (EBC), denaturant effects of sputum processing (dithiothreitol (DTT)), and the limited sensitivity of most detection assays.
Aim We aimed to improve the recovery of inflammatory biomarkers from sputum and EBC from atopic asthmatics. To this end, we applied two alternative processing methods to sputum and sensitive detection techniques in both sputum and EBC.
Methods On two separate visits, EBC and NaCl 4.5%-induced sputum was obtained from 10 non-smoking, clinically stable, atopic asthmatics (4M/6F, 21-54 y, FEV_1 70-122% predicted, $PC_{20}FEV_1$ methacholine<8 mg/mL, SABA *prn* only). EBC was analyzed with antibody based flow-cytometry using Luminex Multi-Analyte Profiling beads. The sputum was processed conventionally, by treatment with DTT to solubilize mucins followed by dialysis to remove DTT (DTT-dialysis) and analyzed with antibody based electrochemiluminescence using Mesoscale multi-array microplates. Subsequently, sputum was obtained from 4 of these patients on two extra occasions: the sample was split manually and half of each sample was processed conventionally and the cell pellet was used as a quality check; the other half was ultracentrifuged (35,000rpm, Ultra) and analyzed with Mesoscale and Luminex.
Results All subjects expectorated analyzable sputum. In DTT-dialysis-processed sputum, spiking experiments with exogenously added cytokines and chemokines showed a poor recovery and almost all biomarkers remained under detection level. After Ultra-processing, several biomarkers were measurable with Mesoscale yielding similar levels on two separate occasions. Hence, Luminex platform analysis was applied allowing detection of a large array of biomarkers. Applying Luminex in EBC, many biomarkers remained below detection limit. In the sputum samples we were able to isolate RNA and perform RNA profiling in the majority of collected samples. Gene expression profiling appeared similar on both visits within subjects.
Conclusion In this exploratory pilot study, we found that recovery of biomarkers was superior in Ultra-processed sputum as compared with DTT-dialysis treated samples, when measured with Mesoscale. Luminex allowed the detection of similar and other biomarkers. In EBC most biomarkers remained below detection limit despite a sensitive detection technology (Luminex). RNA isolation from sputum cells was highly successful and appeared repeatable.

Introduction

Non-invasive sampling methods, including the collection of exhaled breath and sputum analysis, are increasingly applied for assessments of the airway inflammation in clinical monitoring and research in asthma and COPD (1). However, traditional biomarker quantification methods in exhaled breath condensate (EBC) and sputum have yielded conflicting data, due to the lack of standardization of the collection technique (EBC) or to denaturant effects of sputum processing with dithiothreitol (DTT), or to limited sensitivity of most detection assays (2-4).

Quantification of inflammatory mediators in sputum supernatant requires homogenization of the sputum sample with subsequent release of cells and mediators from the mucous bonds. Therefore, DTT is added to the sample to reduce disulphide bonds which cross-link mucinous glycoproteins (5). However, this activity also interferes with the essential disulphide bonds in cytokines and chemokines and hampers the immunological detection of these mediators (5) Recently, Erin *et al,* introduced a dialysis technique to DTT-processed sputum samples, removing DTT after homogenisation and restoring the disulphide bonds, and hence substantially increasing the recovery of cytokines and chemokines (6). Significant differences in cytokine and chemokine levels in supernatant were detected between healthy volunteers and asthmatics, whilst these differences could not be established with the standardized processing.

Ultrasonification or ultracentrifugation is another technique for releasing cells and mediators by physical homogenization which does not require the addition of DTT (7,8). Ultracentrifugation of the entire sample, immediately after collection, yields a homogenous supernatant, devoid of cell debris and mucous. Hadjicharalambous *et al* showed that eotaxin could readily be measured by enzyme linked immunosorbent assay (ELISA) in induced sputum samples from both asthmatics and healthy volunteers following ultracentrifugation whilst this was not feasible in samples (from the same subjects) treated with dithioerythritol (DTE; i.e. an isomer of DTT) (9). A drawback of this technique concerns the disruption of cells and spilling of intracellular content in the homogenate – which of course, can partly account for higher biomarker concentrations (8).

Following adequate sample processing, mediators are mostly measured with immunoassays (3,8). However, several authors failed to demonstrate most of the soluble inflammatory components due to limited sensitivity of these assays (10). Multiplex platforms may offer an advantage due to their capability of simultaneous measurements of up to 100 analytes in small and diluted sample volumes (25-50 ml), such as sputum supernatant and EBC.

The Luminex platforms combine a sandwich immunoassay with fluorescent bead–based detection increasing the specificity and sensitivity of a measurement (11). Gessner *et al* were able to detect a wide range of cytokines in the exhaled breath condensate of COPD patients using a Luminex immunoassay (12). So far, this has been difficult using traditional immunoassays. Mesoscale is another multi-array technology based on chemiluminesence detection for increased sensitivity.

Inflammatory mediators can also be detected at the RNA level in induced sputum after the cells have been lysed (13). Reverse transcriptase polymerase chain reaction (RT-PCR) is a sensitive and specific method for the detection of messenger RNA (mRNA) concentrations even in samples with a low total cell count (14).

In this pilot study we have collected EBC and sputum from a limited number of atopic asthmatic subjects. We aimed to recover RNA from the sputum cells and using microarray analysis conduct gene expression profiling and investigate, via 2-dimensional clustering, the reproducibility of this technique in sputum samples collected on two separate visits in the same subject. The collected sputum supernatant underwent new and optimized processing methods (dialysis and ultracentrifugation) and was analyzed with more sensitive detection techniques (Mesoscale platform, Luminex immunoassay) on two visits to investigate which inflammatory mediators can be measured and assess the reproducibility. In addition, EBC collected in these same subjects also underwent a Luminex immunoassay analysis. With these results the most suitable processing and detection techniques will be selected for a subsequent allergen challenge study in a similar patient population.

Methods

SUBJECTS

Ten non-smoking subjects with clinically stable, mild to moderate persistent allergic asthma were recruited. All patients had a history of persistent asthma for at least 1 year (according to Global Initiative for Asthma (GINA) criteria 15), without any other clinically relevant disorders. Atopy was established by a positive skin prick test to Dermatophagoides Pteronyssinus (ALK-Abello, Nieuwegein, The Netherlands) at screening. Except for stable, infrequent use of inhaled short-acting β2-agonists as required, no one was using concomitant anti-asthma or anti-allergy medication for at least 6 weeks prior to and during the study. Patients had no history of viral infections of the lower airways for at least 3 weeks before enrollment. Caffeine-containing beverages and short-acting inhaled β2-agonists were withheld at least 8 h before

each visit. The study protocol was approved by the Leiden University Medical Centre Ethics Committee, and all participants gave written informed consent.

DESIGN

The study was performed in a single-center setting in two parts (figure 1). In the first part, EBC and subsequently, sputum samples were obtained from 10 eligible, asthmatic subjects on two separate study visits. The sputum was processed according to a novel dialysis technique described by Erin *et al* (6). Following analysis of the sputum samples from the first part, the second part of the study was initiated. Four asthmatic subjects (from the initial 10) returned for two extra sputum inductions (visit 3 and 4). These sputum samples were split in two equal parts by a forceps: one part was processed according to standard procedures and cell differentials were counted as a quality check, while the other part was ultracentrifuged and analyzed, as described below. All the samplings were performed during the same time of the day (±2 hours) on all study visits – and all study visits were separated by 4-10 days.

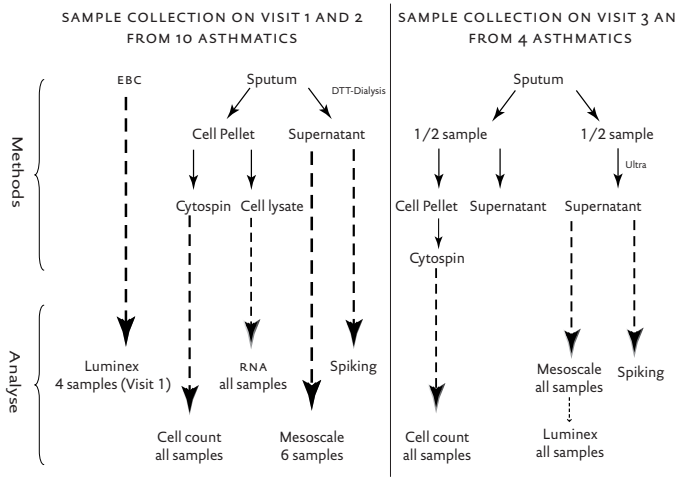

FIGURE 1 Study flowchart

Collection techniques

EXHALED BREATH CONDENSATE COLLECTION

After an acclimatization period of approximately 30 minutes and before sputum induction, each subject was asked to inhale and exhale calmly, while wearing a nose clip, through a mouthpiece and two-way nonrebreathing

valve connected with the EcoScreen condenser (Jaeger, Cardinal Healthcare, Houten, The Netherlands) during a fixed period of 20 minutes (3). Immediately after collection, condensate samples were aliquoted and stored at -80°C pending analysis.

SPUTUM INDUCTION

Sputum induction was performed according to a validated method. Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (Tefa Portanje, Woerden, The Netherlands) and inhaled for 3x5 min at 15 min intervals (16). After each inhalation, or as soon as the subjects experienced cough, they were asked to blow their nose, to rinse their mouth and throat with water, and to expectorate sputum into a clean plastic container by coughing.

Sputum processing techniques

DIALYSIS TECHNIQUE (DTT DIALYSIS)

On study visits 1 and 2 whole sputum samples were processed according to a previously validated protocol by Fahy *et al* (17), with modifications (18,19). Briefly, the volume of the induced sputum samples was determined and mixed with an equal volume of 0.1% sputolysin (Dithiothreitol, Calbiochem, La Jolla, CA, USA). Prior to addition to the sputum, a protease inhibitor tablet (Complete Protease Inhibitor Cocktail tablets, Roche Applied Science #11 697 498 001) was added to 0.1% DTT solution (1 tablet per 50 mL of solution). To ensure adequate homogenization, the samples were placed in a shaking water bath at 37°C for 15 min, once interrupted by gently mixing the sample with a plastic pipette. The homogenized sputum was centrifuged at 1400 r.p.m. at 4°C for 10 min. The sputum supernatant was collected and stored at -80°C until further processing.

After thawing the sputum supernatant 2 μL of 0.5M EDTA and 50 μL of 20% BSA was added per 1 mL of sputum supernatant. Prepared samples were transferred into equilibrated 3,500 MWCO dialysis cassettes (Thermo Scientific cat. no. 66330; 0.5-3.0 mL capacity) with the use of a syringe (Pierce cat. no. 66494). The dialysis cassettes containing prepared samples were placed in a 4 L beaker filled with prechilled Dialysis Buffer 1 and were incubated stirring overnight at 40°C. On the next day, the dialysis cassettes were transferred into a 4 L beaker containing prechilled Dialysis Buffer 2 and were incubated stirring overnight at 40°C. After the double dialysis the sputum supernatants were collected using a syringe and stored at -80°C until Mesoscale and Luminex analysis.

RNA ANALYSIS

After centrifugation and removing the supernatant, the sputum cell pellets were resuspended in PBS to a final volume of 2 mL, followed by filtration through a gauze (pore size 1 mm) to remove clumps. Subsequently, 360 µl was taken for differential cell count. The remaining cell suspension was centrifuged at 4°C for 10 minutes at 390 G (1500 rpm). The supernatant was carefully collected to prevent disrupting of the cells and discarded. The cell pellet was immediately re-suspended in 1ml of cold TRIzol reagent (Invitrogen, Cat. # 15596-018). 1.5 mL of TRIzol was added if the total cell count is > 5×10^6 cells to ensure complete lysis. The solution was mixed by pipetting up and down with a 1 ml pipette, and vortexing. The resulting lysate was stored at -80°C pending RNA analysis.

ULTRACENTRIFUGE (ULTRA)

Four subjects were asked to expectorate sputum on two additional study visits. The sputum samples were carefully divided manually into two portions of equal weight. One part was processed using DTT (as described above) and only the cell pellet was used for cell differentials (as quality control). The other part of the sample was weighed into an ultracentrifuge container (Beckman Coulter 1.5 mL polyallomer microfuge tube; cat. no. 357448 or 3.2 mL glass tube; cat. no. 362333) and 50 µL of a protease inhibitor cocktail was added per 200 mg of weighed sputum. The protease inhibitor cocktail was prepared by dissolving one protease cocktail tablet into 50 mL of 1X PBS (Invitrogen cat. no. 14040). Prepared samples were subsequently ultracentrifuged for 90 minutes in a high-speed ultracentrifuge (Beckman Coulter Inc. Optima Max Ultracentrifuge 130,000 rpm; Fullerton, California) at 35,000rpm (53,500 x g) at 4°C. Sputum supernatants were then collected without disrupting the pellet and stored at -80°C until Mesoscale and Luminex analysis. The pellet was discarded.

Analysis techniques

EXHALED BREATH CONDENSATE ANALYSIS

Biomarkers were measured using the Luminex Human Multi-Analyte Profiles (MAP) immunoassay platform of Rules Based Medicine (Austin, TX, USA) according to manufacturer's directions.

SPUTUM ANALYSIS – SOLUBLE PHASE

To determine the effects of both processing methods on sputum mediators, a recovery analysis was performed by spiking 500 pg of two cytokines, IL-13

(MSD IL-13 Calibrator for Ultrasensitive kit (MSD cat. no. k111ACS-2)) and TNF-alpha (R&D Systems cat. no. 210-TA-050) per 1 mL of sputum prior to incubation at 37°C in the procedure in three randomly selected samples. The spiked analyte levels were within the detection range of the assays. The Mesoscale assays (TNF-α, IL-13, TARC and eotaxin) were performed by electrochemiluminescence (MSD Clinical Development Laboratory, NJ, USA) according to manufacturer's directions.

In samples where biomarkers could be measured using Mesoscale, additional analyses were performed using the Luminex HumanMAP antigen platform of Rules Based Medicine according to manufacturer's directions.

SPUTUM ANALYSIS – CELLULAR PHASE

Cell counts were performed in a haemacytometer (Novex Microscope, The Netherlands), to obtain an estimate of the total number of cells per sample. Subsequently, the samples were diluted with PBS to a final concentration of ± 0.4 x 10^6 cells/mL which was used for preparation of cytocentrifuge slides (50 µL/cytospin; Shandon Cytospin 4, Thermon Electron Corporation, Runcorn, UK). Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells were performed on May-Grünwald-Giemsa-stained cytospins by a certified cytopathologist. In addition, mast cells were counted on Toluidine blue-stained cytospins. In each sputum sample, at least 500 nucleated cells, excluding squamous cells, were counted twice and the average percentage of each cell type was calculated and expressed as percentage nonsquamous cells. If > 80% of the cell count consisted of squamous cells, the quality of the sputum sample was judged unsatisfactory and the entire sample (including the supernatant) was excluded from analysis.

SPUTUM ANALYSIS - GENE EXPRESSION

RNA was isolated from the cell lysate collected on visit 1 and 2 following standard RNA extraction procedures. Total RNA quality (sample integrity) was evaluated utilizing 28S/18S ratio and RNA Integrity Number (RIN) scores obtained from the Agilent Bioanalyzer capillary electrophoresis system. Sample concentration was assessed utilizing the RiboGreen® (Invitrogen) fluorescent dye system. Established pass/fail criteria were then used to identify samples of sufficient quality and concentration for expression profiling. Only specimens that passed the two QC steps were amplified and profiled. These samples were amplified using the NUGEN Ovation amplification technology and profiled on Affymetrix arrays. After scanning, the array data were normalized by multiarray average (RMA) of background-adjusted, normalized, and log-transformed perfect match (PM) values. A profiling QC step was

performed, assessing in particular the percentage of present calls, scale factor, GAPDH and Actin mRNA 5' to 3' ratios for each hybridization, in order to identify potential outliers. Based on this quality check outliers were excluded from subsequent data analysis. The remaining samples were analyzed by 2D unsupervised hierarchical clustering.

ANALYSIS

Only sputum samples with an adequate quality (<80% squamous cells and 500 nucleated cells counted) were included into analysis. The least detectable dose (LDD) was determined as the mean + 3 standard deviations of 20 blank readings. Group sizes in this pilot study were too small for statistical analysis, hence, descriptive statistics was used to describe the results.

Results

EBC ANDSPUTUM QUALITY ASSESSMENT

EBC was obtained from all subjects. All ten subjects were able to expectorate adequate (<80% squamous cells and 500 nucleated cells counted) sputum samples on all visits.

SPUTUM SUPERNATANT AND EBC

Spiking showed a poor recovery for the sputum samples obtained on the first two study visits and processed with the DTT-Dialysis technique (Table 1). Therefore, Mesoscale analysis was randomly performed in 6 samples only. Almost all cytokines and chemokines appeared below the least detectable dose (LDD) (Table 2) and hence, the remaining samples were not analyzed. In the second part of the study, we collected sputum samples from 4 of the 10 asthmatics on two extra visits. Following processing with the Ultra technique, these samples showed a much higher recovery in the spiking experiments (Table 1). Subsequently, these samples were analyzed with the Mesoscale platform (Table 2). The positive results provided support to additional analysis with Luminex. The relevant detectable mediators for inflammatory airway disease are summarized in Table 3. In the same subjects EBC (collected on Visit 1) was also analyzed with Luminex (Table 3).

RNA RECOVERY FROM SPUTUM CELLS

In part 1 of the study, from 17 out of 20 collected sputum samples (10 subjects; 2 visits) sufficient RNA could be extracted from the cell pellet lysate. Specimens collected from the same patient at different times were co-clustered in a dendogram as presented in Figure 2. These data show that total

RNA extraction and profiling of samples obtained on both study visits (visit 1 and 2) were reproducible with variation in the gene expression profiles being smallest within the individual patient.

TABLE 1 PERCENTAGE RECOVERY OF SPIKED IL-13 AND TNF-α MEASURED BY MESOSCALE

PROCEDURE	IL-13 (PG/ML)	TNF-α (PG/ML)
DTT-Dialysis technique (n=3)	27.9 % (± 11.6)	51.6 % (± 4.3)
Ultra technique (n=3)	110.6 % (± 11.3)	62.4 % (± 6.2)

Values are presented as mean (± SD)

TABLE 2 LEVELS OF INFLAMMATORY MEDIATORS IN SPUTUM SUPERNATANT FOLLOWING DTT-DIALYSIS AND ULTRA-PROCESSING MEASURED BY MESOSCALE

PROCEDURE	SUBJECT	EOTAXIN-3 (PG/ML)	TARC (PG/ML)	IL-13 (PG/ML)	TNF-α (PG/ML)
DTT-Dialysis technique	Nr 1 - Visit 1	<LDD	<LDD	<LDD	<LDD
	Nr 1 - Visit 2	<LDD	<LDD	<LDD	<LDD
	Nr 4 - Visit 1	147	<LDD	<LDD	<LDD
	Nr 5 - Visit 1	<LDD	<LDD	<LDD	<LDD
	Nr 5 - Visit 2	<LDD	<LDD	<LDD	<LDD
	Nr 8 -Visit 1	129	12.2	<LDD	<LDD
Sputum Ultra technique	Nr 7 - Visit 3	14.5	<LDD	<LDD	1.74
	Nr 8 - Visit 3	216	20.5	<LDD	5.89
	Nr 9- Visit 3	641	14.3	0.79	25.8
	Nr 10 - Visit 3	37.9	<LDD	<LDD	<LDD
Sputum Ultra technique	Nr 7 - Visit 4	<LDD	<LDD	<LDD	1.57
	Nr 8 - Visit 4	24.4	10.5	<LDD	1.91
	Nr 9 - Visit 4	4892	59.5	3.2	40
	Nr 10 - Visit 4	71.2	<LDD	<LDD	<LDD
<LDD		9.6	9.6	.59	1.52

Mesoscale analysis was randomly performed in 6 samples only on Visit 1 and 2. Four asthmatics returned for Visit 3 and 4 for an extra sputum induction. LDD = Least detectable dose

TABLE 3 LEVELS OF INFLAMMATORY MEDIATORS IN SPUTUM SUPERNATANT AND EXHALED BREATH CONDENSATE MEASURED BY LUMINEX.

PROCEDURE	SUBJECT	α1 ANTI (UG/ML)	α2 MACRO (UG/ML)	ENA-78 (NG/ML)	ENDO-1 (PG/ML)	EOTAXIN (PG/ML)	GLUT-S-TRANS (NG/ML)	IL-13 (PG/ML)	IL-16 (PG/ML)	IL-6 (PG/ML)	IL-8 (PG/ML)	MCP-1 (PG/ML)	MMP-2 (NG/ML)	MPO (NG/ML)	TIMP-1 (NG/ML)	TNF-α (PG/ML)
Sputum Ultra technique	Nr 7 - Visit 3	1.04	1.98	0.60	7.63	17.50	0.14	<LDD	87.20	4.96	1350.00	21.60	44.90	84.90	182.00	1.18
	Nr 8 - Visit 3	0.83	11.50	2.89	7.63	26.80	0.14	<LDD	1900.00	50.70	7720.00	230.00	60.10	150.00	316.00	3.65
	Nr 9- Visit 3	1.00	3.20	2.43	9.13	26.70	0.19	<LDD	2200.00	151.00	16400.00	206.00	59.40	98.30	271.00	12.40
	Nr 10 - Visit 3	0.65	12.90	0.79	20.80	26.30	0.13	<LDD	64.50	3.11	507.00	39.90	48.80	42.20	109.00	0.46
Sputum Ultra technique	Nr 7 - Visit 4	2.16	1.96	0.55	3.81	44.50	0.14	<LDD	126.00	8.12	2730.00	71.70	14.80	731.00	267.00	2.18
	Nr 8 - Visit 4	2.78	10.70	2.08	1.57	56.10	0.17	<LDD	734.00	30.70	3990.00	347.00	19.60	2830.00	415.00	2.74
	Nr 9- Visit 4	2.36	<LDD	2.03	5.14	199.00	0.20	<LDD	3880.00	298.00	33200.00	471.00	30.60	6260.00	>533	49.70
	Nr 10 - Visit 4	2.99	3.75	1.57	20.20	154.00	0.16	<LDD	348.00	11.20	2030.00	200.00	31.50	855.00	351.00	1.45
EBC	Nr 7 - Visit 1	<LDD	<LDD	0.01	6.75	26.60	0.18	<LDD	<LDD	<LDD	1.59	4.31	9.72	<LDD	<LDD	1.81
	Nr 8 - Visit 1	<LDD	<LDD	0.01	8.26	22.50	0.18	<LDD	<LDD	<LDD	2.62	4.66	12.30	<LDD	0.13	1.96
	Nr 9- Visit 1	<LDD	<LDD	0.02	5.78	22.50	0.20	<LDD	<LDD	<LDD	2.10	6.67	8.25	<LDD	<LDD	1.19
	Nr 10 - Visit 1	<LDD	<LDD	<LDD	6.03	22.50	0.15	<LDD	<LDD	<LDD	<LDD	3.61	6.78	<LDD	<LDD	1.35
<LDD		5.3E-05	0.30	0.02	1.44	8.20	0.08	11.38	13.24	2.44	0.70	10.40	30.00	1.36	0.04	0.80

Four asthmatics returned for Visit 3 and 4 for an extra sputum induction. In the same subjects EBC (collected on Visit 1) was also analyzed. LDD = Least detectable dose

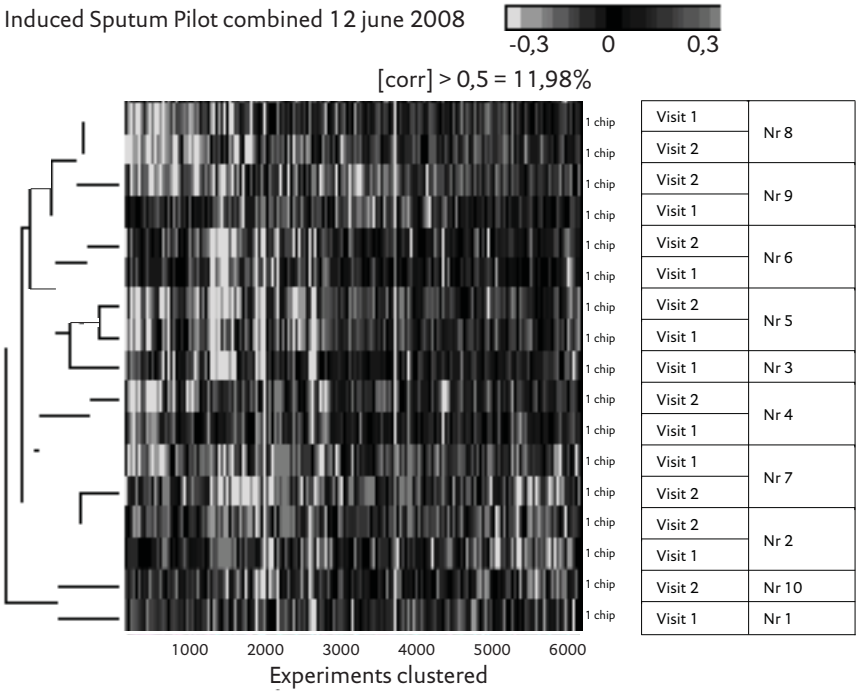

FIGURE 2 Two-dimensional clustering of MRNA collected from asthmatic subjects on study visit 1 and 2.

Discussion

This pilot study was aimed to improve the recovery of inflammatory biomarkers obtained with non-invasive airway sampling techniques, EBC and induced sputum – both from the soluble and the solid phase. To this end we tested two alternative sputum processing methods and applied novel sensitive detection techniques to both the sputum and EBC samples. Applying Mesoscale analysis on sputum samples, processed by the dialysis technique previously introduced by Erin *et al*, we were unable to measure relevant cytokines and chemokines (6). Therefore, this processing technique was not pursued any further, and we processed sputum supernatant with the DTT-free Ultracentrifuge technique described previously (9). In these samples we found a good recovery of spiked cytokines and chemokines, and we were able to quantify several cytokines and chemokines. In addition, in these samples we were able to measure multiple inflammatory mediators with the

Luminex analysis. In contrast, applying Luminex analysis to the EBC samples of these subjects, most mediators remained under LDD. Regarding the solid phase-biomarkers: RNA isolation from sputum cells was highly successful. Seventeen out of 20 collected samples passed the quality control tests and the gene expression profiling appeared similar on both visits within subjects.

In contrast to the DTT-Dialysis, applying the Ultra technique, we were able to quantify several inflammatory mediators in sputum samples. We decided to specifically measure eotaxin-3 with the Mesoscale, since a study by Berkman *et al* reported that eotaxin-3 - and not eotaxin - was upregulated in bronchial biopsies and bronchoalveolar lavage fluid in atopic asthmatics following inhaled allergen challenge (20). We found similar levels of eotaxin-3 measured with Mesoscale and eotaxin measured with Luminex as previously reported by Hadjicharalambous following ultracentrifugation of sputum samples of patients with mild to moderate persistent asthma (9). The authors applied this technique because in their study spiked sputum samples showed low recovery following treatment with DTE without any increase following ultrafiltration. These findings are comparable to our results applying the DTT-Dialysis technique. Interestingly, in the same study, eotaxin levels were not higher following ultracentrifugation (13,000g) compared to regular speed centrifugation (400 g). However, it is unlikely that the Ultra technique yields similar data as the DTT processing technique, since ultracentrifugation causes intracellular contents to spill into the supernatant and consequently may increase the levels of the inflammatory markers. In addition, applying this technique, cell counts cannot be performed due to the cellular disruption by the high speed centrifugation. This can be overcome by splitting the samples, so in one part cell counts can be performed, for the purpose of quality control of the entire sample, while the remaining part is used to investigate the presence of DTT-sensitive biomarkers. Although the numbers were small in the second part of the study, the techniques appeared to yield reproducible results. Patients with the highest biomarker levels on visit 3, presented with the highest levels on visit 4 and in the same range for several detectable mediators (Table 2). A similar pattern was seen in the Luminex analysis (Table 3). Obviously, more precise data on the reproducibility of these detection techniques need to be obtained in larger patient and sample numbers in a more standardized manner. In this setting, the Ultra technique should also be compared to the standardized sputum processing methods (including DTT), and not only to the DTT-Dialysis technique.

Despite the sensitive detection technique employed, a wide range of biomarkers remained below the LDD in the Ultra samples and even more so in the EBC. In agreement with the results by Bayley *et al* in a population with COPD, we were also unable to measure α-antitrypsin, IL-8 and myeloper-

oxidase in EBC (21). Possibly, these biomarkers are present in higher concentrations in other asthma-phenotypes or under different conditions, e.g. in a more severe disease or following an exacerbation, rather than in stable disease. For example, Csoma *et al* found higher levels of leukotrienes in EBC in patients with severe asthma than in patients with moderate persistent asthma, while levels in mild asthmatics were similar to healthy controls (22). In addition, several studies reported increased levels of leukotrienes following an asthma exacerbation as compared to stable disease (22,23).
The large array of mediators in the Luminex platform is ideal to provide an impression of which inflammatory mediators are present in a sample and hence, can help to decide on what biomarkers to focus. Subsequently, the Mesoscale platform that was specifically developed at a collaborating laboratory to measure mediators in sputum can reliably quantify these mediators.

Most studies have employed RT-PCR to focus on the expression of several genes of interest (13,24). In this pilot study, we are amongst the first to perform global genes expression profiling in sputum cells. We were able to recover suitable RNA in the majority of samples and the profiles were fairly similar on visit 1 and 2 in the same subjects. Again, RNA expression profiling is probably more valuable in an intervention study whereby the profiles before and after the intervention (e.g. allergen challenge or drug administration) can be compared. For example, a recent study reported a clear enhancement of expression patterns involved in airway inflammation following an endotoxin challenge in allergic individuals compared to pre-challenge expression levels (25).

In conclusion, we compared two alternative processing techniques of induced sputum in combination with two sensitive biomarker detection techniques to improve biomarker quantification from non-invasive sampling techniques. The DTT-dialysis technique did not yield measurable levels of inflammatory mediators in sputum samples. Applying the Ultra technique, we were able to measure several cytokines and chemokines in sputum and also in EBC employing the broad Luminex platform and sensitive Mesoscale analysis. However, many mediators still remained below the LDD. In the same sputum samples we were able to isolate RNA and perform expression profiling. This technology provides a viable platform for identification of specific signatures between patients. These techniques will be applied and further validated in an interventional allergen challenge study in our institute. These findings may open a pathway to identify a magnitude of potential biomarkers even in small volume-samples.

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