Analysis of Short and Long Term Variability of Nasal Mucus versus Breath Condensate Inflammation Markers in Healthy Individuals

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Abstract

Objective: Inflammation markers were measured in nasal mucus and exhaled breath condensate (EBC) to assess the normal variability of these markers among healthy individuals. The measurement of protein markers in those matrices is valuable to provide information on inflammation endotypes and local reaction of the immune system.

Methods: Inflammation markers (IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α) in nasal mucus and EBC from six healthy adults were measured by Meso Scale Discovery technology. Nasal mucus was collected with sponges of polyurethane foam, which is a recently developed sampling technique. EBC was sampled via an optimized breath manoeuver with the RTUBE™ device. Both collections were done within a limited sampling time of two minutes. The samples were taken on the same day and within a time period of twenty days for the determination of short and long term variability, respectively.

Results: All inflammation markers were detectable in nasal mucus. This was in contrast with the measurements in EBC, in which only IFN-γ, IL-8 and IL-13 were detectable in the majority of the samples. EBC collected with the applied breathing technique gave a repeatable almost constant volume (448 μL (95% CI: 429 to 467 μL)). The collected volume of nasal mucus was more variable (from 0 to 200 μL). The variability of nasal analytes was similar over the short and long time period. The nasal inflammation marker levels were more stable within an individual than between different individuals (0.72 ≤ ICC ≤ 0.96). This was in contrast with the measurements in EBC that were highly variable over time (0.08 ≤ ICC ≤ 0.51).

Conclusion: We indicate – given the good detectability and low variability of the inflammation markers determined in nasal mucus – that nasal mucus, more than EBC, is potentially a good matrix to assess inflammation in the respiratory system.

Keywords: Nasal mucus; Polyurethane foam; Exhaled breath condensate (EBC); RTube™; Inflammation; Intra-class correlation coefficient (ICC)

Introduction

The measurements of protein markers in samples of the nasal, upper and lower airways are valuable to provide information on inflammation endotypes and local reaction of the immune system. The nose and bronchi show the same immune response to allergens causing inflammation of the airways [1-3]. During an inflammatory response, inflammatory cells like dendritic cells, macrophages, mast cells, basophils, eosinophils, neutrophils, B and T lymphocytes are activated and mediators such as leukotrienes, prostaglandins, histamine and cytokines are secreted [4-6]. These mediators are required for the communication between cells and they act on target cells to induce cellular functions such as activation, proliferation, growth, cell differentiation, apoptosis, chemotaxis and secretion of other mediators [7-9]. Cytokines can be divided into different groups with certain functions [10]: (a) pro-inflammatory cytokines that strengthen the inflammation, e.g. interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF); (b) anti-inflammatory cytokines that weaken the inflammation, e.g. IL-10, interferon (IFN)-γ, IL-12, IL-18; (c) lymphokines that are predominantly secreted by T cells and regulate the immune response, e.g. IL-2, IL-3, IL-4, IL-5, IL-13, IL-15, IL-16, IL-17; (d) growth factors which promote the survival of cells and provide the structural changes in the airways, e.g. transforming growth factor (TGF)-β; and (e) chemokines with chemoattractant properties that help the recruitment of inflammatory cells to the site of inflammation, e.g. IL-8.

Various methods are available for sample collection of airway cell secretions. Invasive techniques such as bronchoalveolar lavages and biopsies are risky and not always well tolerated. There is a need for further exploration of non-invasive techniques such as sampling of nasal mucus and exhaled breath condensate (EBC) which can be applied to easily monitor large patient groups or study populations [11]. Nasal mucus is composed of 95% water, 2% mucin, 1% salts, 1% other proteins (such as albumin, immunoglobulins) and 1% lipids [12]. It can be collected via lavage techniques, but sponges of e.g. polyurethane foam are useful for undiluted sampling [12-14]. In the current study we used sponges of polyurethane foam to collect nasal mucus on the epithelial surface. Exhaled breath consist of a gas phase and a vapor phase. EBC is obtained by cooling the vapor phase of the breath which contains aerosol particles. EBC is composed of 99% of water and 1% of nonvolatile compounds (such as cytokines, lipids, ions), and volatile water-soluble compounds (such as ammonia, hydrogen peroxide, ethanol) [15]. Research on EBC has been done since several years, but there is still room to improve the method of sampling [15-

18]. We sampled EBC via an optimized breath manoeuver [19] with the portable collection device RTube™ [20]. Using these state-of-the-art methodologies the variability of inflammation markers within and between healthy individuals were determined to study their stability within one day and over a longer time period of twenty days.

Methods

Study population and study design

Six healthy adults 20-50 years old were recruited in April and May 2014. We did not set out any criterion for inclusion or exclusion of the participants in this study considering allergies and common colds. An even amount of men (N=3) and women (N=3) were included. All the subjects gave written informed consent to participate in the study. The study was approved by the ethical committee of the University of Antwerp (ID number: B300201316329).

This study had two parts: a short term and a long term follow-up. In the short term experiment nasal mucus and EBC were sampled from three subjects (three women) at six different time points on the same day between 8 a.m. and 6 p.m. In the long term experiment nasal mucus and EBC were sampled from six subjects on five different days in a twenty day period, always in the morning between 9 a.m. and 11 a.m. We did not restrict food intake by the participants in the time frame before the sampling, as this was not expected to influence inflammation marker levels.

Sampling method

Collection of nasal mucus: Sterilized sponges of polyurethane foam (Gummi Welz, Neu-Ulm, Germany) were used for the collection of nasal mucus. A sponge (20 x 15 x 5 mm) was placed in each nostril of the subject for two minutes. Both sponges were then placed in a 0.22 μm cellulose acetate Spin-X filter in a 2 mL centrifuge tube (Corning, USA). Centrifugation was done at 4500 rcf for 10 minutes at 4°C. The resulting nasal mucus was transferred with Maximum Recovery Tips (Axygen, Tewksbury, Massachusetts, USA) to a 1.5 mL Protein LoBind Eppendorf tube (Eppendorf, Hamburg, Germany). These special tips and tubes were used to avoid proteins from interacting with the plastic surface so loss of proteins would be minimal. The samples were stored at -80°C until further analysis.

Collection of exhaled breath condensate: The portable sample device RTube™ (Respiratory Research, Inc., Austin, Texas, USA) was used for the collection of EBC. Subjects were breathing through the RTube™ for two minutes in a controlled way: in cycles of one second of inhalation and three seconds of deep exhalation according to the protocol of Johnson and Morawska [19]. No forced breathings (coughing, sneezing) were allowed. The exhaled air was cooled by means of an aluminum cylindric condenser that was cooled at -20°C for at least 30 minutes prior to sampling. The resulting EBC was transferred with Maximum Recovery Tips to a 1.5 mL Protein LoBind Eppendorf tube. The samples were stored at -80°C until further analysis.

Inflammation markers analysis

All samples of nasal mucus and EBC were analyzed with Meso Scale Discovery (MSD) Proinflammatory Panel 1 (human) kit (Meso Scale Discovery, Rockville, Maryland, USA). From this 10-spot multiplex kit six inflammation markers were measured: IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α. The MSD technology is based on multiplex sandwich enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescent detection. From the collected nasal mucus 90 μL was used for the analysis. In case there was not enough sample,

nasal mucus was diluted with demineralized water. Thereafter 90 μL of Diluent 2 of the MSD kit was added, resulting in at least 1/2 dilution. From the collected EBC 180 μL was used for the analysis. Thereafter 90 μL of Diluent 2 was added, resulting in 2/3 dilution. The standards and nasal mucus samples were analysed in duplicate using 50 μL per well. For the EBC samples three wells were used, each one containing of 75 μL. The rest of the procedure was performed according to the protocol of the MSD kit. The 96-well MSD plates were incubated while shaking at room temperature for three hours. After washing, 25 μL of detection antibody solution was added to each well. The plates were further incubated while shaking at room temperature for two hours. After washing, 150 μL of 2X Read Buffer T was added to each well. Afterwards, the plates were immediately read at 620 nm using a MESO QuickPlex SQ 120 (Meso Scale Discovery, Rockville, Maryland, USA). The standard curve range of the assay was 0.12 - 510 pg/mL for IL-8, IL-1β, and IL-13; 0.08 - 325 pg/mL for IL-10 and TNF-α; 0.3 - 1230 pg/mL for IFN-γ. The theoretical detection limits of respectively IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α were 0.20, 0.04, 0.04, 0.03, 0.24 and 0.04 pg/mL. Samples below the detection limit were given half of the value of the lowest calculated concentration.

Data analysis

Descriptive values such as mean, standard error, minimum, lower quartile (P_{25}), median (P_{50}), upper quartile (P_{75}) and maximum were calculated for all measurements in EBC and nasal mucus. The normality of the inflammation marker levels was checked using the Shapiro-Wilk's W test. The natural logarithm was taken to obtain a normal distribution of the data. A repeated measures analysis of variance (ANOVA) was performed with STATISTICA version 10 (StatSoft, Inc., Tulsa, Oklahoma, USA) to calculate the intra-class correlation coefficient (ICC) of the ratio of between-individual variance divided by the sum of between-individual and within-individual variance. The ICC gives an indication of the variability of the repeated measurements within one individual. ICC values were interpreted as following: < 0.40 weak agreement, 0.40 - 0.75 fair to good agreement and > 0.75 excellent agreement [2,21].

Results

Collected volume of nasal mucus and EBC

In total 45 EBC and 42 nasal mucus samples were collected from six healthy individuals. The sampling methods were well tolerated by all participants.

The volume of nasal mucus collected for two minutes with nasal sponges varied greatly, with yields between 0 and 200 μL. Three collections resulted in no nasal fluid sample. The coefficient of variation for all the collected volumes of nasal mucus for the short time experiment was 44.91%. For the long term experiment this was 97.11%. When the collected volume of nasal mucus was too low for the analysis, the sample was diluted with demineralized water.

A relatively constant volume of EBC was obtained with the RTube™, independently of the individual being sampled. After two minutes of breathing in a controlled way (in cycles of one second of inhalation and three seconds of deep exhalation according to the protocol of Johnson and Morawska [19]) in the RTube™, the mean collected volume of EBC was 448 μL (95% confidence interval (CI): 429 to 467 μL). The coefficient of variation for all the collected volumes of EBC for the short time experiment was 15.21%. For the long term experiment this was 13.53%.

Descriptive values of inflammation markers in EBC and nasal mucus

The concentrations of the inflammation markers in EBC were very low and many were below the detection limit. IFN-γ, IL-8 and IL-13 were detectable in the majority of samples (Table 1). In nasal mucus all inflammation markers could be easily detected in undiluted as well as diluted samples (Table 2). The concentration of nasal IL-8 was not quantifiable due to concentrations above the highest standard (>529.1 pg/mL).

Short term variability

All samples of nasal mucus and EBC were collected for all three female volunteers. The variability of repeated measurements of nasal mucus on the same day was low, in contrast to the measurements in EBC that were highly variable. The ICCs of nasal mucus inflammation marker measurements were all excellent (Table 3), which means that the agreement of measurements within a person was higher than between the individuals. The ICC of EBC inflammation marker measurements were weak (Table 3), only IL-8 levels in EBC were more stable. ANOVA couldn't be performed for measurements of IL-1β and IL-10 in EBC and IL-8 in nasal mucus, because concentrations were respectively below or above the standard curve range.

Long term variability

EBC was collected in all individuals. Three samples of nasal mucus were missing: two for person 6 (male) on the third and fifth day and one for person 5 (male) on the fifth day. The variability of repeated measurements of nasal mucus for the twenty day period was low, in contrast to the measurements in EBC that were highly variable. The ICC of nasal mucus inflammation marker measurements were all excellent (Table 3), which means that the agreement of measurements within a person was higher than between the individuals. The ICC of EBC inflammation marker measurements were weak (Table 3), only TNF-α levels in EBC were more stable. ANOVA couldn't be performed for measurements of IL-1β in EBC and IL-8 in nasal mucus, because concentrations were respectively below or above the standard curve range.

The ICC values of short and long term nasal analyte levels were similar (Table 3), except for IFN-γ that had a better stability on the same day than on the long term. On the opposite, IL-13 had a better stability on the long term compared to the within-one-day follow-up.

Discussion

Measuring inflammation markers in nasal mucus and EBC can provide valuable information about inflammation endotypes and local reaction of the immune system [11,22]. Due to a strong functional and immunological relationship between the nose and the bronchi, the inflammatory response in the nose is similar to the situation in the lungs [1-3].

Nasal mucus can be collected in order to measure inflammation markers in the nasal airways [11]. The nose is one of the first places of contact with the outer environment and is therefore an interesting tissue. The nasal mucosa doesn't only come in contact with pollen, but

Analyte	Mean (±SE)	Min	P25	P50	P75	Max
IFN-γ	0.0451 (±0.0090)	0.0011	0.0011	0.0178	0.0764	0.2624
IL-1β	0.0033 (±0.0012)	0.0008	0.0008	0.0014	0.0014	0.0446
IL-8	0.0244 (±0.0032)	0.0026	0.0056	0.0204	0.0329	0.0865
IL-10	0.0036 (±0.0009)	0.0002	0.0002	0.0019	0.0045	0.0373
IL-13	1.1944 (±0.0947)	0.1715	0.7667	0.9803	1.8007	2.4779
TNF-α	0.0075 (±0.0012)	0.0002	0.0002	0.0063	0.0114	0.0275

Table 1: Descriptive values for all 45 collected EBC samples: mean ± standard error (SE), minimum (Min), lower quartile (P25), median (P50), upper quartile (P75) and maximum (Max) in pg/mL per analyte.

Analyte	Mean (±SE)	Min	P25	P50	P75	Max
IFN-γ	207.6 (±130.1)	0.4	2.0	14.1	32.2	5250.5
IL-1β	155.4 (±64.1)	3.7	15.5	40.1	105.5	2521.9
IL-8	>529.1	>529.1	>529.1	>529.1	>529.1	>529.1
IL-10	6.6 (±1.3)	0.3	1.3	3.3	7.9	39.8
IL-13	43.9 (±7.5)	1.5	18.0	28.6	52.2	291.6
TNF-α	12.1 (±1.8)	0.5	3.0	7.7	18.8	51.9

Table 2: Descriptive values for all 42 collected nasal mucus samples: mean ± standard error (SE), minimum (Min), lower quartile (P25), median (P50), upper quartile (P75) and maximum (Max) in pg/mL per analyte. The theoretical detection limit of respectively IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α were 0.20, 0.04, 0.04, 0.03, 0.24 and 0.04 pg/mL.

Analyte	Short term variability		Long term variability	
	ICC nasal mucus	ICC EBC	ICC nasal mucus	ICC EBC
IFN-γ	0.96	0.08	0.72	0.26
IL-1β	0.96	/	0.90	/
IL-8	/	0.44	/	0.19
IL-10	0.87	/	0.84	0.23
IL-13	0.77	0.32	0.87	0.33
TNF-α	0.93	0.23	0.89	0.51

Table 3: The calculated intra-class correlation coefficient (ICC) of the measurements in nasal mucus and EBC per analyte for the short and long term follow-up. /: below or above standard curve range

also with bacteria, viruses, particles, air pollutants and irritants [2]. Sponges of polyurethane foam are recently used as collectors for nasal mucus. Riechelmann et al. [13] showed how the use of these sponges was superior to other tested techniques (nasal lavage, nasal spray blow technique and filter paper) for recovery of inflammation markers [13]. In the current study inflammation markers (IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α) were measured in nasal mucus collected with this type of sponges. This sampling method was well tolerated by all participants. However, placement of a foreign substance for a period of time may traumatize the mucosa and alter concentrations of analytes under investigation [13,14]. In our study we repeated the sampling every two hours during one day and we didn't observe any increase in analyte levels. Because of the short contact time of two minutes and the inert nature of the material used, there was little chance of traumatization of tissue or irritation. The concentrations of nasal analytes were all easily detected and all within or above the range of the standard curve. Good detectable levels of markers in nasal mucus are certainly an advantage compared to those in EBC. Nasal secretions can be collected without dilution, whereas the inflammation marker levels in EBC are 10 000 times lower than the concentrations in lung lining fluid [11,17,18]. The measured concentrations of nasal inflammation markers in the current study were of the same order or higher than those reported in other studies [1,13,14,23]. The concentrations of the nasal inflammation markers were approximately 1000 times higher than the concentrations in EBC. McDougall et al. [24] and Pringle et al. [25] reported that some nasal epithelial cells' mediators such as IL-8 and IL-6 had an increased constitutive expression compared to bronchial epithelial cells [24,25].

A disadvantage of the nasal mucus collector was the highly variable collected volume per person and per sample time point. This is possibly related to the inter-individual differences in morphology and accessibility of the nasal mucosa at the height of the nasal septum and the placement of the collector. The volume of collected nasal mucus after two minutes varied between 0 to 200 µL. If too little volume is collected, the nasal fluid can be diluted with a known factor [1,12-14]. We have applied this method for some samples, but we didn't test the influence of *ex vivo* nasal mucus dilution on the measured concentration of inflammation markers. Alternatively, more sample of nasal mucus could be pooled from repeated samplings on the same day since we showed a low variability within an individual. However this methodology was not tested in this study.

EBC can be collected in order to measure inflammation markers in the lungs, although there is some discussion on its reproducible collection. The sampling of EBC is simple, non-invasive and safe [15-18]. When breathing normal for 5 to 15 minutes, approximately 1 mL of EBC can be collected [15]. In the current study an optimized breathing technique was tested, which is based on the mechanism of breath aerosol formation by Johnson and Morawska [19]. They showed that the aerosol production was higher in case of a fast inhalation and deep exhalation [19]. In the current study the participants had to breath for two minutes in the RTube™ in a controlled way, i.e. in cycles of one second of inhalation and three seconds of deep exhalation. This optimized breathing technique would be more difficult to apply with young children, who do not have the needed breathing coordination skills. However, in the current study with adults, a sufficient and fairly constant volume of EBC (448 µL (95% CI: 429 to 467 µL)) could be collected for analysis in a very short time. Since the RTube™ has the disadvantage that the condensation temperature can vary during sampling [16], shortening the sampling time was supposed to increase the stability of the EBC analyte levels. However, the concentrations of

the inflammation markers (IFN-γ, IL-1β, IL-8, IL-10, IL-13 and TNF-α) were low and often below the detection limit. On the other hand, as IFN-γ, IL-8 and IL-13 were detected, EBC could be an appropriate matrix for some inflammation markers. The measured concentrations of EBC inflammation markers were lower than or equal to other reported literature values. Different collectors, analysis methods and/or sample processing procedures could explain these differences [9,26-28]. IL-1β in EBC was of the same order of previous measurements in children in which EBC was sampled using the RTube™ and analysed in our lab [20].

The measurements in EBC were highly variable over time (0.08 ≤ ICC ≤ 0.51), except for the relative stability of IL-8 during one day (ICC=0.44) and TNF-α over a twenty day period (ICC=0.51). The higher variation of analytes in EBC was possibly due to a higher technical variability of measuring in the lower range of the MSD method.

The variability of nasal analytes was similar over the short and long time period. The nasal inflammation marker levels were more stable within an individual than between different individuals (0.72 ≤ ICC ≤ 0.96). IFN-γ had a better stability within the same day than within the long term period. IFN-γ is a Th1 cytokine and is known to be expressed in e.g. common colds [4,7,26]. Therefore it can be expected that IFN-γ is more variable over a longer period. Unexpectedly, IL-13 had a better stability in the long term follow-up than on the same day. IL-13 is a Th2 cytokine which plays a role in isotope switching to Immunoglobulin E (IgE) production and is important for sensitisation [4,6,7,26,29]. The correlation between inflammation markers was tested with Spearman rank correlation. The nasal levels of inflammation markers were all correlated (see Supplementary information: Table S.I), except for IFN-γ (Th1 cytokine) and IL-13 (Th2 cytokine) indicative for their different role in inflammation. We didn't observe a correlation between inflammation markers in nasal mucus and EBC. We speculate that this is due to the low detectability of inflammation markers in EBC.

Some limitations of this study were the limited number of participants, and the loss of statistical power because of non-results due to dry sponges. We also did not investigate the relationship between short and long term information on disease/infection, allergen exposure or pollutants and the respective fluctuation of inflammation markers. Certainly, reference and relevant clinical values need to be established based on larger study populations performing allergen challenges and longitudinal sampling post exposure. A clinical relevant increase of a factor 10 for e.g. IL-13 and INF-γ was observed by Scadding et al. [14], who performed a nasal challenge with purified Timothy grass allergen. These high levels were not seen in the current study population.

Conclusion

Undiluted nasal mucus was collected in a simple way using polyurethane sponges. In case the sampled mucus volume was too low, a larger amount could be obtained by repeated sampling on the same day because of the low variability of nasal inflammation markers within an individual. The fact that the nose is a first contact tissue of e.g. allergens, combined with nasal inflammation markers being more stable over time and easily detectable, makes nasal secretion - more than breath condensate - a potentially good matrix to assess inflammation in the respiratory tract.

Acknowledgments

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