



## Early View

Research letter

# Missing sputum samples are common in asthma intervention studies and successful collection at follow-up is related to improvement in clinical outcomes

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Please cite this article as: Frøssing L, Hvidtfeldt M, Silberbrandt A, *et al.* Missing sputum samples are common in asthma intervention studies and successful collection at follow-up is related to improvement in clinical outcomes. *ERJ Open Res* 2022; in press (<https://doi.org/10.1183/23120541.00612-2021>).

This manuscript has recently been accepted for publication in the *ERJ Open Research*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJOR online.

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**Title:**

Missing sputum samples are common in asthma intervention studies and successful collection at follow-up is related to improvement in clinical outcomes

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**Conflicts of interest:**

The authors have no conflicts of interest to declare.

**Take home message:**

Several factors significantly impact ability to produce a sputum sample after an anti-inflammatory intervention and we argue that the widely used complete-case analysis is inappropriate for paired sputum-based outcome measures.

**Word count: 1193 (1200)**

## Manuscript

With only modest agreement between airway- and systemic eosinophilia, biomarkers directly assessing the level and type of airway inflammation are becoming increasingly important, both for targeting treatment to the individual patient, as well as for assessing effect (1).

A sputum cell differential count remains the gold standard for airway inflammometry in asthma but missing data are an inherent issue when utilizing sputum-based outcome measures as the success rate for induction of ranges markedly (2,3). It is well known that missing data potentially can lead to substantial bias when inadequately handled (4). At present, reflections on how to handle missing sputum samples are largely absent in the literature and most studies defer to the use of baseline values to predict outcomes or utilize complete-case analysis despite evidence highlighting multiple imputation as the superior statistical method independent of the missingness of the data (2,3,5–8).

With the advances in the feasibility of sputum sampling in a clinical setting; we foresee a marked increase in the utilization of sputum-based outcome measures highlighting the necessity to evaluate the missingness of induced sputum (9,10).

We hypothesized that any patient's ability to produce a sputum sample after receiving medical treatment was not random and that the proportion of missing samples were higher in patients with response to treatment as a result of resolution of airway inflammation in general and the IL-13 driven mucus hypersecretion in particular (11). Therefore, we pooled data on mannitol induced sputum from 3 intervention studies (n=135) with an aim to identify predictors of successful induction prior and post a medical intervention.

The RECONSTRUCT study was a single-arm intervention study (1600 $\mu$  inhaled budesonide once daily for 16 weeks) of steroid-free asthma patients with airway hyperreactivity to mannitol (ClinicalTrials.gov identifier: NCT03034005).

The UPSTREAM study was a placebo-controlled intervention study (add-on of anti-TSLP(n=20) or placebo(n=20) for 12 weeks) of predominantly moderate-to-severe asthma patients with airway hyperreactivity to mannitol (12).

The SIGNATURE study was a single-arm intervention study (add-on of 37.5mg oral prednisolone for 2 weeks) of patients with moderate-to-severe asthma(7).

Successful sputum induction was not required for inclusion in any of the studies and maintenance treatment prior to enrollment was continued unchanged throughout the study period.

In all three studies, sputum was collected following a mannitol challenge test in a specimen jar (Petri dish) with the sample quality continuously evaluated by a trained lab technician. Samples were processed using the plug selection method processed, and cut-off values for eosinophilia and neutrophilia were  $\geq 3\%$  and  $\geq 61\%$ , respectively (7,12).

Three-fourths (75%, n=101) of patients were able to produce a sufficient sputum sample at baseline, two-thirds (65%, n= 88) at follow-up and paired samples were collected in half of the patients (52%, n=70). Success rate for collection of sputum at follow-up was equal in the placebo group and in the patients receiving active treatment and we found no significant difference in the success rate between baseline and follow-up across all patients, in patients receiving active treatment nor in each study individually.

At baseline, neither demographics, lung function, airway hyperreactivity (PD15 to mannitol) or inflammatory profile (blood eosinophils, FeNO, IgE and atopy) were significantly related to a successful collection.

In the patients receiving active treatment, success rate at follow-up was significantly higher in patients without ICS at baseline (80% vs. 57%, OR 4.3, p= 0.006) and significantly lower in patients receiving treatment with LABA (42% vs 72%, OR 0.27, p= 0.003) and LAMA (9% vs. 26%. OR 0.29, p= 0.02) at baseline (Figure 1). Similarly, patients with severe asthma according to ERS/ATS criteria had a significantly lower success rate compared to those without (54% vs. 77%, OR 0.31, p=0.008) (13).

For patients receiving active treatment, those with the paucigranulocytic inflammatory phenotype at baseline had a significantly lower success rate at follow-up (41% vs. 59%, OR 0.46, p=0.05).

Successful collection at follow-up was not associated with other baseline inflammatory markers (sputum eosinophils, blood eosinophils and FeNO) nor the reduction in these..

Across all patients, successful collection of sputum at follow-up was significantly more prevalent in patients with improvement in FEV1 ( $\Delta$ FEV1 200mL and 12%) and decreased airway hyperreactivity at follow-up measured with a mannitol challenge test (79% vs 57%, OR 2.4, p=0.02 and 82% vs 58%, OR 3.3, p=0.01 respectively).

In the patients receiving active treatment, this remained significant for mannitol (p=0.01) and showed a strong trend for FEV1 (p=0.08).

We did not identify any factors affecting the success rate of induction at baseline; however, the likelihood of a successful induction post intervention decreased with higher maintenance ICS doses at baseline and was - in line with our hypothesis - lower in the absence of airway inflammation (paucigranulocytic sputum). Surprisingly, a clinical response to treatment – with increase in lung function or improvement in AHR – was associated with a higher likelihood of a successful induction post intervention. Speculatively, this could be explained by relief of airway obstruction which in turn allows for a mobilization of distal airway mucus plugs.

Interestingly, the level of maintenance treatment and the anti-inflammatory add-on intervention exerted opposite effects on the success rate. We believe this to be explained by the fact that they reflect different aspects (traits) of disease: maintenance ICS dose is reflective of disease severity (i.e. chronicity) whereas response to add-on treatment reflects reversibility of disease. A notion, we believe to be supported by the increase in success rate at follow up in the steroid naïve patients in the RECONSTRUCT study (figure 1).

Mannitol induced sputum samples are of good quality and are comparable with samples induced with hypertonic saline (HT) for the analysis of inflammatory cells and soluble markers (14,15). Further, the success rate for mannitol- and HT induction are similar, and the success rates reported in this study are equal to our own prior efforts using saline and to previous reports using mannitol (3,14).

Still, we note several factors that potentially influence the generalizability of our findings to HT induced samples, and we believe future studies confirming our results in HT saline induced samples are warranted. Wood et al reported a significantly lower total cell count ( $3.8$  vs.  $2.1 \times 10^6$ ,  $p=0.003$ ) in samples induced with mannitol compared with HT saline (14) which we believe may hamper our generalizability as we defined successful induction based on total cell count. Further to this point, Wood et al and Alvarez-Puebla et al have both reported significant differences in inflammatory phenotype classification using mannitol- and HT saline induction respectively which; as we found the paucigranulocytic phenotype to be significantly associated with a lower success rate at follow-up; again hampers the generalizability of our results (14,15).

Successful induction was defined based on identification of  $\geq 250$  cells and acknowledge that other cut-offs or criteria – such as viability – could have been chosen; however, as no consensus exists this task is inevitably difficult and biased.

In summary, our findings suggest that the ability to produce repeated sputum is not random and should be taken into account in the planning and analysis of interventions studies.

Based on our findings, we believe that complete-case analysis is inappropriate for paired sputum-based outcome measures. We speculate that imputation better accommodates the missingness of sputum, but future studies evaluating different methods for handling missing data are warranted (8).

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