



Early View

Original research article

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Mucosal Cryobiopsies – A new Method for Studying Airway Pathology in Asthma

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Take home message

The cryotechnique is a promising safe tool for obtainment of mucosal biopsies in patients with asthma yielding larger and better-preserved specimens compared with forceps biopsies increasing utility for evaluating airway pathology

Abstract

Background

In vivo studies of airway pathology in obstructive lung disease are limited by poor quality of specimens obtained with forceps. Obtainment of cryobiopsies has increased diagnostic yield in cancer and interstitial lung disease but has not been used in patients with asthma. In a recent pilot study, we found mucosal cryobiopsies to be larger and more intact than conventional forceps biopsies.

Aims and Objectives

To compare quality and safety of mucosal cryobiopsies versus conventional forceps biopsies in patients with asthma.

Methods

Endobronchial biopsies were obtained with forceps and cryoprobe from patients with asthma not currently treated with inhaled steroids and evaluated histologically.

Results

A total of 240 cryobiopsies and 288 forceps biopsies were obtained from 48 patients. Bleeding from the biopsy site was common but self-limiting. No major complications related to the procedure were seen. Cryobiopsy cross areas were 4 times larger compared with forceps. Stretches of intact epithelium were detected in in all cryobiopsies compared to 33% in forceps biopsies. Further, the length of intact epithelium was on average 4 times longer in the cryobiopsies. Importantly, there was a good preservation of both antigens and mRNA in the cryobiopsies ensuring a suitability and robustness for immunohistochemistry and in-situ hybridization.

Conclusion

Obtainment of mucosal cryobiopsies in patients with asthma is safe and yields biopsies that are significantly larger and morphologically better preserved compared with traditional forceps biopsies. The cryotechnique thus seems to be a promising tool for future *in vivo* studies of airway pathology.

Word count: 245

Key Words: Asthma, bronchoscopy, cryobiopsy

Introduction

Asthma is a heterogeneous disease where understanding underlying disease mechanisms and applying clinically observed phenotypes to specific endotypes remains a challenge(1). Since asthma is associated with both immunological and structural changes in most compartments of the airways including the epithelium, submucosal glands and airway smooth muscle, sampling at the site of disease is highly important to further unravel underlying pathology. Historically, endobronchial biopsies has been an important tool in such mechanistical research and bronchoscopy in patients with asthma is generally safe, even in patients with severe disease(2–6). In vivo studies of airway pathology are, however, often limited by the poor quality of specimens that can be obtained with endobronchial forceps. Biopsies are small with crushing artifacts and only limited content of deeper lying structures such as airway smooth muscle(7,8). Importantly, novel advanced techniques for studying pathology in airways disease in depth, such as single cell sequencing, require larger and more intact airway samples(9). Clearly, there is a need for better methods for obtaining high quality samples, to further our understanding of disease mechanisms in obstructive airways diseases.

In suspected lung cancer, the use of a cryoprobe to obtain biopsies from both flat and exophytic endobronchial lesions has in recent years shown to be both safe and superior to forceps(10–12). The use of transbronchial cryobiopsies has in parallel increased diagnostic yield in supposed interstitial lung disease and decreased the need of the more invasive video assisted thoracoscopy(13,14). In a pilot study of patients with hemoptysis investigating the use of mucosal cryobiopsies to study airway histology, we showed that the method yields bigger biopsies with better preservation of the tissue sampled as well as higher contents of airway smooth muscle and submucosal glands(15).

Safety is paramount when using bronchoscopy as a research tool and studies on transbronchial cryobiopsy for diagnosing interstitial lung disease report an increased risk of pneumothorax of up to

30% and bleeding as a common complication that needs to be handled e.g. with a Fogarty balloon. Further, studies on endobronchial cryobiopsy in cancer diagnostics report an increase in non-severe bleeding compared to sampling with forceps(11,12,14). This raises a concern regarding safety of the method as a research tool in patients with asthma. In the above-mentioned pilot study, one patient had a severe bleeding needing intervention from both the site of forceps and cryobiopsy, but the study was not powered to evaluate safety. Further, biopsies were not performed in patients with airway disease where mucosal vascularization and fragility may be altered. Hence, we sought to further validate the usefulness and safety of bronchial cryobiopsies in a larger number of patients with inflammatory airway disease.

In the present study, we examined the use of mucosal cryobiopsies in patients with asthma, with the aim to compare the quality of mucosal cryobiopsies with conventional forceps biopsies, and to evaluate safety of performing mucosal cryobiopsies.

Methods

The RECONSTRUCT study is a single center clinical prospective intervention study examining disease mechanisms in patients with asthma at Bispebjerg University Hospital in Copenhagen, Denmark. Patients underwent bronchoscopy before and after a 6-week course of high dose inhaled corticosteroids. Airway mucosal biopsies were obtained using cryotechnique and standard forceps and evaluated histologically at the University of Lund, Sweden. To assess airway pathology and the quality of airway specimens without the effect of steroids, we enrolled steroid-free asthma patients with airway hyperresponsiveness to mannitol. (ClinicalTrials.gov identifier: NCT03034005).

Population and ethics

In order to assess airway morphology without the effect of steroids, we enrolled patients with asthma not treated with neither inhaled nor oral corticosteroids for at least the past three months, who had signs of active asthma with airway hyperresponsiveness to mannitol (PD15<635mg).

Exclusion criteria were:

- FEV1<70% of predicted
- active smoking or a history of smoking with more than 10 pack years
- competing respiratory diseases including lower respiratory tract infections within the past 4 weeks
- significant comorbidity (ASA<2)(16)
- pregnancy or breastfeeding
- hypersensitivity to inhaled corticosteroids
- uncontrolled hypertension
- acute myocardial infarction within past 6 months
- aorta or cerebral aneurisms
- recent abdominal operation
- failure to comply with the study protocol in general

All Participants were given oral and written information on the aims, contents and risks of the study and given the opportunity to bring an assessor if needed. All patients gave oral and written consent to participate in the study.

The study was approved by the Danish National Committee on Health Research Ethics (H-16043663), the Danish Medicines Agency (EUDRACT: 2016-03509-33) and the Danish Data

protection Agency (BFH-2018-018) and monitored by the Danish Good Clinical Practice Unit (Project ID: 2016-898). ClinicalTrials.gov identifier: NCT03034005.

Bronchoscopy and tissue sampling

Bronchoscopy was performed as a standard outpatient procedure with a flexible bronchoscope (Olympus BF-1TQ180/BF-1TH190, Olympus, Hamburg, Germany).(17) The sedatives Midazolam and fentanyl were administered according to the operator in doses ranging from 5-20 mg and 50-150 µg respectively. A single dose of 1000 mg tranexamic acid was administered in the beginning of the procedure as bleeding prophylaxis. Oral access with bronchoscopic guided placement of an orotracheal tube (BronchoFlex®, Rüsch, GA, USA) was used for easy access and retrieval of the bronchoscope during sampling with the cryoprobe as well as for protection of the vocal cords.

Supplemental oxygen was administered through a designated canal in the tube as needed.

Mucosal biopsies were obtained with both forceps (Endojaw™, Ø 1.9mm, Olympus, Hamburg, Germany) and cryo-technique (Erbecryo® 2, Ø 1.9mm, Erbe, Tübingen, Germany). The technique used for obtainment of cryobiopsies has been described elsewhere.(15) Briefly: Mucosal cryobiopsies were obtained from longitudinal stretches at subsegmental levels at the discretion of the operator. The cryoprobe was placed adjacent to the airway wall before initiating a freeze cycle of 2-3 seconds where after the probe and bronchoscope was gently pulled and retracted. Biopsies were immediately thawed in a saline bath, transferred to fixative and subjected to overnight fixation in 4% buffered formaldehyde before routine dehydration and paraffin embedment. Bleeding was scored as: none, mild, moderate or severe according to British Thoracic Society guidelines.(17)

Histological Analyses

We used standard histological techniques to evaluate the size of the biopsies and to explore morphology. The preservation of mucosal structural integrity and architecture was evaluated by the

presence and length of intact epithelium stretches at the biopsy border. Multiple 3 µm tissue paraffin sections were generated from the paraffin-embedded biopsies. Sections stained with standard hematoxylin-eosin were digitized by an Aperio slide scanner (Aperio AT2, Aperio, buffalo Grove, IL, USA) and used for morphological assessment and quantitative morphometric analysis. Separate serial sections were also evaluated for suitability to perform routine protein visualization by immunohistochemistry as well as visualization of specific gene expression (mRNA) by in situ hybridization.

For immunohistochemistry, antigens were retrieved in a pretreatment module (PT-Link) machine (DakoCytomation, Glostrup, Denmark) before tissue sections were subjected to single and double immunostaining by an automated immunohistochemistry robot (Autostainer Plus, DakoCytomation, Glostrup, Denmark) and polymer horseradish peroxidase linked secondary antibodies (EnVision™ G2 Doublestain System Rabbit/Mouse, K5361, Dako) using diaminobenzidine (DAB) and Vina green as detection chromogens.(18) The following primary antibodies were used to test stain for selected structural and immunological cells: anti-alpha smooth muscle actin (clone 1A4, Dako, dilution 1:200), anti-human cluster of differentiation 31 (CD31, found in human endothelial cells) (clone JC70A, Dako; dilution1:50), anti-human interleukin-33 (IL-33, an epithelial alarmin) (clone Nesy-1, Alexis; dilution 1:500), anti-human mast cell tryptase (clone Mab 1222A, Millipore; dilution 1:10 000), mouse anti-human cluster of differentiation (CD3, found on T-cells) (clone 2.38, Dako; dilution 1:50), anti-human cluster of differentiation 207 or Langerin (CD207, found in Langerhans cells) (langerin, Leica; dilution 1:100). All primary and secondary antibodies used for immunohistochemistry have been extensively validated for use in clinical diagnostics and research. For in situ hybridization, messenger RNA (mRNA) probes against the human house-keeping gene ubiquitin C and the prokaryote gene DapB were used as positive and negative mRNA controls, respectively. The probes were visualized using the RNAscope 2.5 HD detection kit Brown assay kit

(Advanced Cell Diagnostics, Hayward, CA, USA) according to the instructions supplied by the manufacturer.(19,20) Sections were incubated with endogenous enzyme block, boiled in pre-treatment buffer and treated with protease before target probe hybridization. The target RNA was then amplified using a series of amplification solutions and visualized with colored chromogen.(19,20)

Statistical analyses

Descriptive statistics were performed. Results are presented as medians with interquartile ranges and numbers with percentages. For comparison of size and histological quality parameters of biopsies, means with 95% CI are presented and tests for differences were performed using paired T-tests.

All analyses were performed using IBM® SPSS statistics version 22 (IBM Software, IL, US).

Results

Population

Fifty patients were included in the study. Two patients withdrew consent before the first bronchoscopy due to personal reasons. A total of 5 mucosal cryobiopsies and 6 forceps biopsies were obtained from 48 patients resulting in a total of 240 and 288 biopsies, respectively. Clinical characteristics are presented in Table 1.

Biopsy size and tissue preservation

Mean cross section area of the cryobiopsies were approximately 4 times larger compared with forceps biopsies: 2.05 mm² (95% CI: 1.71-2.39) versus 0.50 mm² (95% CI: 0.32-0.68) corresponding to a difference of 1.55 mm² (95% CI: 1.16-19.4), P<0.001, see figure 1.

Stretches of intact epithelium was detected in all cryobiopsies compared with in 33% of the forceps biopsies. The mean length of intact epithelium in cryobiopsies was approximately 4 times longer compared with forceps biopsies: mean length 653 μm (95% CI: 567-653) versus 166 μm (95% CI: 77-255) corresponding to a difference of 487 μm (95% CI: 368-606), $P < 0.001$; see figure 1.

Representative micrographs of the cryobiopsies are presented in figure 2.

Because of the larger biopsy size, it was also evident that the cryobiopsies had larger areas of cross-sectioned subepithelial lamina propria tissue, airway smooth muscle bundles and subepithelial glands.

Immunohistochemistry and in situ hybridization

The cryobiopsies were evaluated for their suitability for detection of protein cell markers and mRNA expression with immunohistochemistry and in situ hybridization, respectively. Paraffin sections from cryobiopsies displayed good intensity of immunoreactivity (figure 2). Likewise, mRNA detection by in situ hybridization resulted in clear and robust staining, as evidenced by staining for negative and positive control mRNA probes (figure 2).

Safety

Mild bleeding from the biopsy site after cryobiopsy was a common complication seen in all patients. There were no cases of severe complications including moderate or severe bleeding, pneumothorax, or procedure-related pneumonia. All patients were discharged the same day after standard observation in the recovery ward without any post-procedural complications. Two patients (4%) withdrew from the study after the bronchoscopy due to discomfort related to the procedure.

Time needed for each biopsy was not recorded specifically but it is the opinion of the operators that this does not differ notably between the two techniques, approximately around 5 minutes for a total

of 5 cryobiopsies. Preparation and placement of the orotracheal tube does increase procedural time slightly.

Discussion

This study has for the first time showed that the obtainment of endobronchial cryobiopsies in patients with asthma yields specimens of better quality compared with biopsies obtained with forceps. Cryobiopsies are bigger and better preserved can be used at standard outpatient setup without a noteworthy increase in procedural time-consume and importantly without introducing significant risk of severe complications for the patients. We believe that this technique can set a new standard for obtainment of airway specimens ensuring biopsies of better quality to increase applicability and improve opportunities in future in vivo studies of asthma.

The current study is the first study to evaluate the potential of cryobiopsies for studying airway pathology in airway disease. We have showed that biopsies obtained with the cryotechnique yields bigger biopsies with better preservation of the morphology. The results are in line with results from our previous study in patients with hemoptysis as well as what has been published on endobronchial cryobiopsies in other fields e.g. suspected lung cancer(15,21). Mucosal cryobiopsies in this study were on average 4 times larger than biopsies obtained with forceps. Endobronchial cryobiopsies obtained from malignant lesions have in some circumstances shown to be even bigger(21). It is our experience that extending freezing cycles beyond 3 seconds results in difficulties of retracting the cryoprobe, supposedly because of involvement of underlying cartilage structures which we believe limits the size of biopsies obtained. If the cryoprobe was undetachable we have successfully stopped the freezing cycle and waited until the frozen area has thawed before initiating a new cycle. All cryobiopsies contained stretches of intact epithelium whereas this was only the case in 33% of the forceps biopsies. In comparison a quality study by Labonté et al. showed that intact epithelium

was only present in 41% of forceps biopsies(7). Indeed unintended mechanical desquamation of the epithelium is a common crushing artifact present in specimens obtained with forceps from both patients with airway disease and healthy controls(22). A reason for a slightly lower rate of forceps biopsies containing intact stretches of epithelium in the current study could be more strict evaluation criteria applied. In the current study the epithelium should be firmly attached to the basement membrane and with preserved tight junctions before considered intact. Nevertheless, this does not change the main message regarding a comparison of epithelial preservation between forceps and cryobiopsies, where stretches of intact epithelium were not only longer but also present in all specimens obtained with the cryo-method. The more preserved epithelium is not only due to larger biopsy size. The main reason could be the fact that once frozen, the epithelium and other tissue structures are spatially fixated, and this prevents the type of elastic tissue-damaging stretching that may occur during the withdrawal of traditional forceps after closing the jaws.

Safety was a big concern and mild bleeding from the cryobiopsy site was seen in all patients. Bleeding was, however, self-limiting in all instances and we saw no other procedure-related adverse events. A low complication rate was also the finding in our pilot study in patients with hemoptysis but has now been validated after obtainment of 240 cryobiopsies(15). A similar safety profile has been observed in a trial with 600 patients with suspected lung cancer where the rate of severe bleeding needing intervention after obtainment of cryobiopsies was comparable to what was seen after forceps biopsies(11). In this study, systemic tranexamic acid was administered for bleeding prophylaxis. The evidence behind the use of tranexamic acid for this purpose is limited though a recent study showed less bleeding after transbronchial lung biopsy when tranexamic acid was instilled locally.(23) Considering the low complication rate seen in this study, the need of prophylactic tranexamic acid for prophylaxis is uncertain and needs further investigation.

Pulmonary arterial hypertension is generally a contradiction for performing transbronchial cryobiopsy as it may increase risk of bleeding.(14) In this study, pulmonary arterial pressure was not evaluated in line with other studies evaluating endobronchial cryobiopsies in cancer diagnostics. Whether Pulmonary arterial hypertension increase the risk of bleeding when obtaining endobronchial cryobiopsies needs therefore to be further investigated.(11)

Sedatives needed to perform the procedures were within standard dosing regimens as also reported previously(11,15).

Despite a few minutes extra preparation-time placing the protective orotracheal tube, the cryomethod does not increase procedural time compared with obtaining biopsies with forceps as has also been demonstrated for endobronchial sampling with the cryoprobe in patients with suspected lung cancer where time spent obtaining each biopsy was estimated to be approximately 5 minutes for both forceps biopsies and cryobiopsies(11).

Importantly, freezing artifacts was not a problem for neither standard histological evaluation nor cellular and molecular explorations by immunohistochemistry or in situ hybridization. That said, the cryo-procedure will inevitably cause mechanical damage in a micro scale due to ice crystal formation. For this reason, cryobiopsies cannot be used for ultrastructural electron-microscopic studies.

Since it is not possible with standard bronchoscopes to retract the cryoprobe along with the bronchoscope without such placement to protect the vocal cords and ensure easy access and retrieval, an orotracheal tube is mandatory increasing procedural time slightly. In rare events of uncontrolled bleeding or other emergencies, the orotracheal tube on the other hand may become an advantage ensuring easy introduction of e.g. tamponade balloons or suction catheters for establishing bleeding control.

The current study was performed in patients not currently treated with inhaled corticosteroids, presumably with most patients having mild to moderate disease. Though safety of performing research bronchoscopies in patients with severe disease does not seem to be increased safety and applicability of the cryomethod in these patients need to be further validated(3).

Being able to obtain bigger biopsies from the airways without increasing procedural risk, increases applicability and utility for studying airway disease. We believe that the ability to perform in vivo studies investigating pathology and response to e.g. treatment with specimens of better quality that are not affected by the sampling process itself can push our understanding of underlying disease mechanisms in asthma further. With biopsies containing a high degree of well-preserved epithelium and deeper lying structures the opportunity to investigate these compartments as secluded entities or perhaps more importantly as complex networks improves. Obtaining cryobiopsies from the airways can therefore complement further research in both the important role of the airway epithelium in maintaining both health and airway disease and airway smooth muscle(24). Indeed, studying airway smooth muscle pathology in asthma has been limited due to the low content in forceps biopsies increasing both numbers of study participants and the number of specimens obtained from each individual to make meaningful investigations(8). The need of well-preserved large biopsies is also evident when using more novel techniques including transcriptomics and single cell sequencing(25). Whether mucosal cryobiopsies can be used for this needs further exploration though. While post-mortem samples can give valuable information, studies including such samples remain to be of observational character limiting the ability to draw causal conclusions and improving the quality of airway specimens with new sampling techniques to further our understanding of airway pathology through vivo studies are therefore much needed(26).

Besides applicability in a research setting, this new method may also become valuable in clinical practice. Though bronchoscopy is not a part of the standard diagnostic approach in airway disease,

it may be needed in specific cases with diagnostic challenges or severe refractory disease.(27) With increasing need of assessing underlying disease drivers and sub-phenotyping patients before initiating targeted treatment we suspect this need to increase in the future.

Conclusion

This study shows that mucosal cryobiopsies obtained from patients with asthma are both bigger and better preserved than biopsies obtained with forceps. Importantly the method is safe and can be performed at a standard outpatient setup. The method seems therefore to be superior to forceps with the potential to improve in vivo studies and our understanding of airway pathology in asthma.

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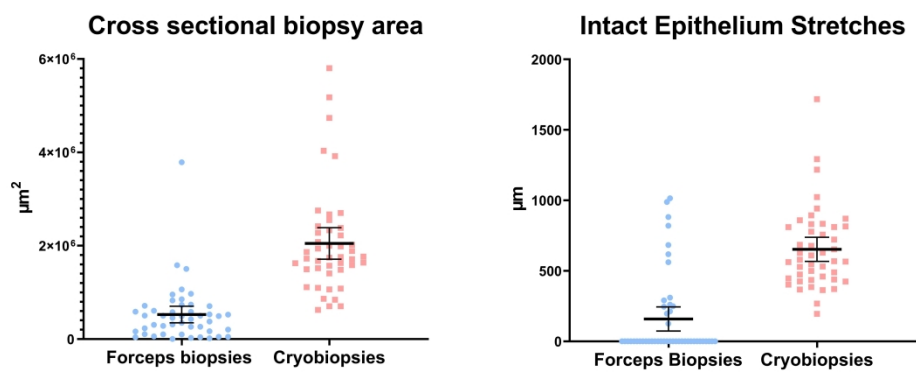
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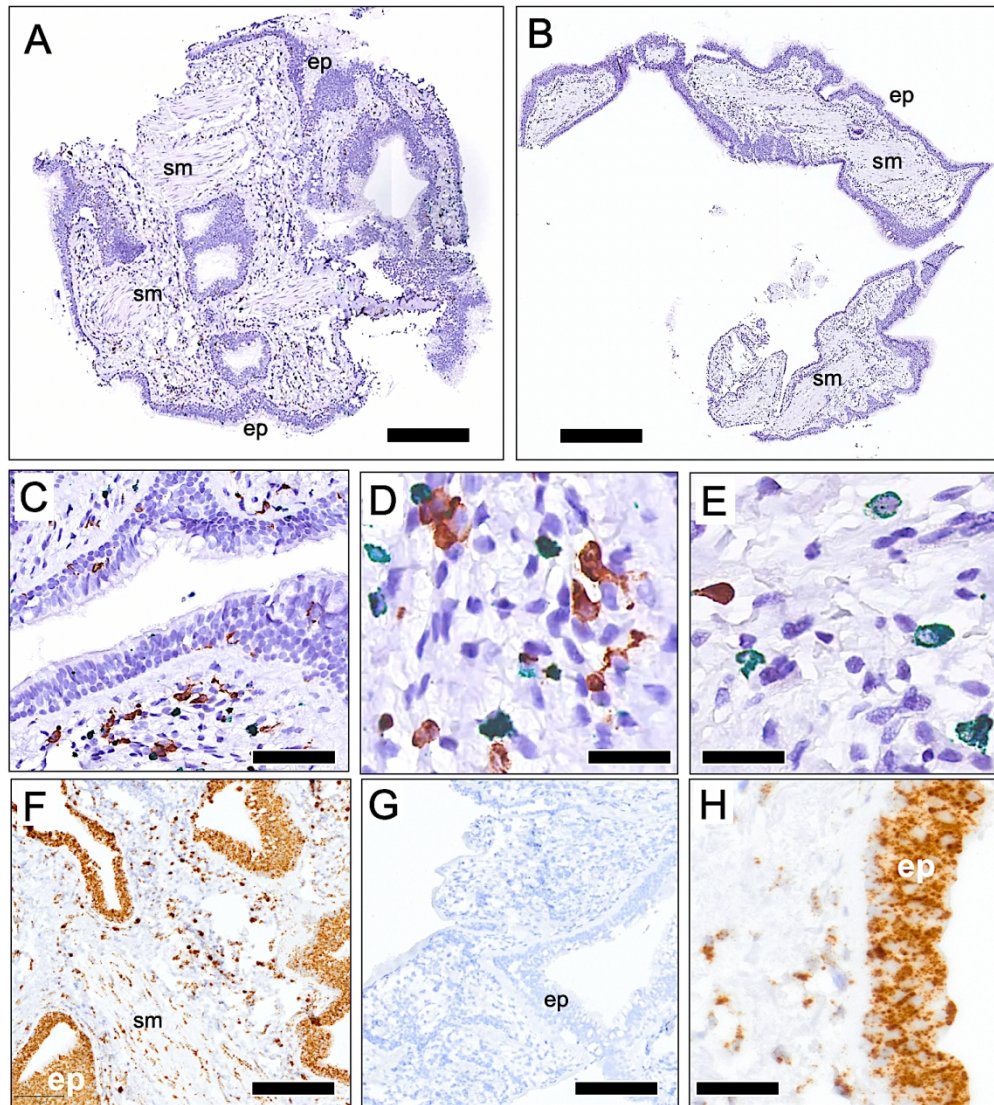
Tables

Table 1 Clinical characteristics presented as numbers with percentages and medians with interquartile range.

Participants, n	48
Mucosal cryobiopsies, n	240
Forceps biopsies, n	288
Age, y	25 (7)
Female participants, n	24 (50%)
BMI, kg/m ²	23.4 (4.7)
ACQ-5 score	1.42 (1.33)
FEV ₁ , L	3.77 (1.46)
FEV ₁ , % predicted	99 (15)
FEV ₁ /FVC	0.77 (0.12)
PD15 Mannitol, Mg	185 (337)
FeNO, ppb	27.4 (46.7)
Former smoker, n	9 (19%)
Age at asthma onset, y	12.5 (19)
Atopy, n	28 (58%)



Cross sectional biopsy area (A) and length of intact stretches of epithelium in forceps biopsies (blue) vs. cryobiopsies (red). Individual measures with mean and 95% CI.



(A-B) Representative overview micrographs of cryobiopsies from asthmatic bronchi. (C-E) Examples of robust immunohistochemical (IHC) identification of airway mucosal immune cells (C-D: green, MPO+ neutrophils; brown, CD68+ macrophages, panel E: green MCT and brown MCTc mast cells). (F,H) Detection of the positive control gene UBC mRNA in situ hybridization (ISH). (G) Negative ISH control with probe against the soil bacteria gene DapB. Ep = epithelium, sm = smooth muscle. Scale bars; A=250um; B, 350 um; C, 70um; D-E, 25um; F-G, 120um; H,50 um.