Author’s response to reviews

Title: Nerve ablation after bronchial thermoplasty and sustained improvement in severe asthma

Authors:
Nicola Facciolongo (facciolongo.nicola@ausl.re.it)
Antonino Di Stefano (antonino.distefano@icsmaugeri.it)
Vladimiro Pietrini (vladimiro.pietrini@unipr.it)
Carla Galeone (Galeone.carla@asmn.re.it)
Maria Federica Bellanova (mariafederica.bellanova@unipr.it)
Francesco Menzella (Menzella.Francesco@asmn.re.it)
Nicola Scichilone (nicola.scichilone@unipa.it)
Roberto Piro (Piro.Roberto@asmn.re.it)
Gianluigi Bajocchi (Bajocchi.gianluigi@asmn.re.it)
Bruno Balbi (bruno.balbi@icsmaugeri.it)
Lorenzo Agostini (Agostini-Lorenzo@asmn.re.it)
Pierpaolo Salsi (Salsi.pierpaolo@asmn.re.it)
Debora Formisano (Formisano.Debora@asmn.re.it)
Mirco Lusuardi (Lusuardi.Mirco@ausl.re.it)

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Author’s response to reviews:

To Prof. Peter B Noble
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Nerve ablation after bronchial thermoplasty and sustained improvement in severe asthma.

Authors: Nicola Facciolongo (corresponding author); Antonio Di Stefano; Vladimiro Pietrini; Carla Galeone; Maria Federica Bellanova; Francesco Menzella; Nicola Scichilone; Roberto Piro; Gianluigi Bajocchi; Bruno Balbi; Lorenzo Agostini; Pierpaolo Salsi; Debora Formisano; Mirco Lusuardi

Replies to reviewers’ comments.

Dear Prof. Noble,

We wish to thank you and the reviewers for your comments that have been an invaluable help to significantly improve the paper.

A point-by-point reply to reviewers’ comments is enclosed.

We are confident that now the manuscript is clearer and more complete. A revised version of the article has been submitted to your attention, hoping in a favourable evaluation.

Please don't hesitate to contact us for any further question.

Thank you again and best wishes,

Nicola

[Nicola Facciolongo, corresponding Author]

Reviewer reports:

Howard Mitchell (Reviewer 1):

1) The protocols are a little hard to follow but it appears that there were 3 bronchial thermoplasty (BT) exposures followed by various studies extending 12 months after the last. As well as reviewing the wording of relevant text, the authors should clarify the legends and headings associated with Tables.

Response: table headings and legends have been clarified, with legends in a separate page. We have added more detailed information in the text on BT protocol page 4, lines 13-18 as requested.

2) The statistical treatment is obscure. What are the N values for the histology and C-fibre work? The authors say that 6 serial sections (50 micron thick) were cut from each of 6-8 biopsy specimens. How are these averaged to provide an N value?
Response: presented value for each time point is the highest score obtained from the sections evaluated and not the mean that anyway would have been quite imprecise to calculate from semiquantitative scores. In this sense a potential bias would be unfavourable to the main hypothesis and gives more support to our conclusions.

3) Table 2 (page 16, lines 43-47) says biopsies were from lower lobes. Yet Methods say they were from the carina (page 4, lines 24-25).

Response: We now corrected the text and specified that biopsies were taken from the carina of segmental - subsegmental bronchial lobes at every time points studied.

4) Table 2 (page 16, lines 43-47) indicates that the control biopsy (T0) was taken from the left lung while biopsies at the time periods under investigation (T1, 2 and 12) were taken from the other side of the lung. Without supporting data, the two sides cannot be confidently compared.

Response: Bronchial biopsies taken at a similar airway generation (subsegmental bronchi) show sufficiently comparable morphology and degree of inflammation as demonstrated in previous papers (Gamble E, et al. Eur Respir J 2006;27:293-9, Sont JK et al, Eur Respir J 1997 Nov;10(11):2602-8)). Furthermore, on a theoretical basis we should have submitted patients to a supplemental baseline bronchoscopy to obtain biopsy in the same area to be treated with BT (right lower lobe) on a subsequent endoscopy procedure. We have chosen an ethical compromise to limit the number of bronchoscopies to the standard BT protocol.

5) Shouldn't measurement (biopsy, clinical) happen just before the next BT rather than immediately after, as stated in Methods (page 4, lines 24-25)?

Response: Clinical evaluations were not carried out immediately after BT (see table 2): at baseline before the first BT session and 3 and 12 months after a BT session. Biopsies were taken concomitantly with BT application in the contralateral lung. This protocol was adopted also in major large trials on BT (see references 5-7).

6) PGP9.5 was used to determine C-fibres. PGP9.5 is a pan neuronal marker. In that case it will reveal the rich cholinergic innervation of the airway as well as everything else. How then is the C-fibre identified?

Response: we used an incorrect terminology, since we had no means to phenotype nerve fibers (as stated by referee 2 C-fiber is a functional definition). Therefore in text now we refer simply to nerve fibers.

7) The heading of Table 5 (page 20) says several structural measures (basement membrane thickness, disepithelization degree, endothelium) are presented yet only the basement membrane thickness is shown. Did BT ablate the epithelium too and what happens to ASM? Which endothelial cells were studied?
Response: We reported in the text (page 7, line 24) that the degree of epithelial sloughing as well as thickness of the basement membrane and number of endothelial cells was similar at all time points. The antibody anti-CD31 used identifies all endothelial cells in the bronchial biopsies, at variance with peripheral airways where a different antibody needs to be used (anti vW-factor). ASM was not studied in detail, apart from nerve fibers in smooth muscle.

8) The Title, Introduction, Methods prepare the reader for a study focusing on C-fibre, particularly the density in the epithelium, glands and importantly ASM (page 4, lines 55-57). Yet by the Results (page 7, lines 9-15) the authors explain that technical or sampling problems prevented the density of nerves to be determines in most areas of the airway, except for the submucosa. Whilst appreciating the technical difficulties raised by the authors, this limitation restricts the weight of the investigation. Of particular importance would be the density of C-fibres in the ASM layer, since this is the way C-fibres could reduce airway narrowing (see comments elsewhere).

Response: Actually technical problems were found in the evaluation of nerve fibers in glands; in the epithelium fibers were rarely found. As you can see in text data are reported for nerve fibers in submucosa and ASM (see also figure 2 panel b), although a full evaluation of ASM thickness was not carried out.

9) The authors show that effect of BT on C-fibres persists up to 12 months. It is unclear to me how this observation supports the hypothesis that mechanisms other than ASM ablation (eg. C-fibre ablation) are involved in the clinically beneficial effects of BT? Perhaps the ASM is also ablated up to 12 months?

Response: Data in literature confirm persistence of ASM ablation at 3 months (see reference 10), to our knowledge no studies evaluated ASM at 12 months. Our paper is the first demonstrating a persistent ablation of nerve fibers at 12 months. Anyway, whether reduction of ASM function is due to persistence of ASM ablation or reduction of nerves stimulating ASM are both plausible explanation that need further studies. Pretolani et al (ref. 10) demonstrated a significant correlation between ASM and nerve fiber reduction at 3 months with clinical improvement at 12 months. A direct cause-effect relationship between biological and clinical data is anyway still to be confirmed in our opinion.

10) Regarding the rationale for the study and the experimental approach, it is not entirely surprising that BT ablates nerve as well as muscle (as shown by others). Hypothetically, if the muscle is knocked out surely it hardly matters whether the nerves remain intact or not because without some functional muscle to work on the nerves cannot do anything much in terms of airway narrowing. C fibre derived tachykinins cause airway narrowing by activating ASM. The hypothesis that C-fibres and BT are linked only holds water if you can separate the effects of BT on ASM, C-fibres and clinical outcomes.

Response: The concept is sound and we appreciate your point of view but we must admit that we are not able to discriminate the real weight of the different modifications with respect to
clinical improvement. Actually, most papers on BT report only hypothesis on possible correlation between airway modifications induced by BT and clinical data.

11) To this reviewer, the paper addresses inflammatory cellular changes in the airway wall as much as C-fibres. Most Methods, Results and Discussion focus on cellular contributions rather than C-fibres, about which few findings are reported due in part to the technical difficulties alluded to before.

Response: We tried to better balance our discussion considering inflammatory changes and nerve fibers changes reported. We have shortened a bit the part of the discussion related to inflammatory changes.

12) Is there an error in TLC at T3 (Table 4)? Ranges for TLC go up to 80L?

Response: data have been corrected

13) The sequence of endoscopic procedure is shown in Table 2, not Table 3 as stated (page 4, line 26).

Response: corrected

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Brendan James Canning, Ph.D. (Reviewer 2):

Facciolongo and colleagues have studied the effects of bronchial thermoplasty on nerve staining in the airways of patients with severe asthma and related by inference the changes in nerve staining to clinical outcomes. They observed a marked decrease in the number of nerve fibers stained in bronchial biopsies in these patients at 12 months after intervention. In parallel with these anatomical changes, the authors summarized clinical benefits in these patients that seemed to far exceed those reported in several previous studies of bronchial thermoplasty in asthmatics. From these results the authors speculate that ablation of nerves and not ablation of smooth muscle may be the primary therapeutic action of bronchial thermoplasty in asthmatics.

Comments.

1) This is a well written manuscript with a clearly stated objective and with results that are both interesting and consistent with the author's discussion.

Thank you for this encouraging comment.

2) I would dispute the author's assertion that PGP staining is equivalent to identification of C-fiber endings in the airways submucosa. "C-fiber" is a functional definition that is not attainable in a histochemical study, with "C" referring to the slow wave of a conducted compound action potential. Even if the terminal appearance of the nerve endings are consistent with an unmyelinated nerve branch, this could simply reflect a terminal
afferent axon that loses its myelination near its target structures. In fact, there is no clear way of differentiating afferent from efferent nerves in thin sections without additional phenotyping. Within the submucosa there may be arterioles receiving sympathetic adrenergic innervation, or parasympathetic cholinergic or noncholinergic innervation. The stained structures may also be portions of postganglionic axons projecting to structures (e.g. glands) that are not contained in the biopsies. The data as presented are consistent with the author's assertions. But the authors might want to acknowledge that they have not fully phenotyped the stained structures in their biopsies.

Response: we appreciate this expert comment and agree that our description was really incorrect without additional phenotyping that was outside the possibilities of our study. The term C-fibers has been eliminated with reference to our original data and substituted with nerve fibers.

3) Implicit in the narrative of the discussion is that the authors believe that actions on smooth muscle are likely unrelated to any clinical benefits realized in patients receiving bronchial thermoplasty. One scenario mentioned is an effect on neurogenic inflammation. I would question the relevance of inflammation to any of the clinical benefits observed. Neurogenic inflammation is prominent in the airways of rats and guinea pigs but there is scant evidence that anything comparable occurs in human airways. Neurokinins are largely absent from the structures stained by PGP 9.5 in human airways while the account for upwards of 80% of the PGP-IR nerve terminals in the mucosa of mice or guinea pigs. It is also unclear that inflammation in any form leads directly to asthma. Nonasthmatic seasonal allergic rhinitis patients have profound inflammation of their airways in season, and yet, do not have asthma, do not have an increase in reversible airways obstruction and do not develop airways hyperresponsiveness. So if the effects of thermoplasty are not due to effects on inflammation, what could be the explanation? It would be worthwhile discussing further the actions of reflex effects resulting from C-fiber activation. As the authors note, anticholinergics provide significant benefit in asthmatics and this is almost certainly attributable to their actions on smooth muscle regulation, either basal tone or reflex-induced increases in tone. Many irritant stimuli evoke bronchospasm in asthmatics primarily through parasympathetic reflexes. Preventing excessive reflex bronchospasm following C-fiber activation and not an effect on inflammation is a far more likely mechanism for bronchial thermoplasty in severe asthma.

Response: we pointed our attention on a possible mechanism of action of BT different from or complementary to ASM ablation, but we had no intention to consider ASM as irrelevant. We agree on comments about neurogenic inflammation and have eliminated the term form the text. On the other hand we found really modest variations of inflammatory cells and agree that effects of BT on inflammation are unlikely to account for clinical benefits of BT. This has been clarified in discussion.

4) The authors provide clear evidence that lung function was not improved following thermoplasty. What about airways reactivity or responsiveness to bronchodilator anticholinergics?
Response: we did not test airways reactivity or responsiveness to bronchodilator anticholinergics in our study. On the other hand it would have been quite difficult to perform those tests on patients on maximal standard inhalation therapy. A drug wash out might have been detrimental form a clinical point of view, although we agree that on a pathophysiological groud those data would be very interesting.

5) The concept of nerve ablation for obstructive lung diseases is currently being evaluated. The authors should review and cite the following publication: Slebos DJ, Klooster K, Koegelenberg CF, Theron J, Styen D, Valipour A, Mayse M, Bolliger CT. Targeted lung denervation for moderate to severe COPD: a pilot study. Thorax. 2015 May;70(5):411-9.

Response: the reference has been quoted and commented in discussion

6) The clinical benefits observed in this small patient sample seems to exceed that reported previously in several studies of thermoplasty. Would the authors comment on what specifically about their trial or their patients rendered this treatment so effective?

Response: any conclusion is prevented by the very small patient sample as compared with large trials. One possible suggestive explanation is that we performed a number of applications larger than the average in literature (75 versus 50). (see also Langton Respir Research 2017 18:134). The point has been added in discussion.

7) The authors report clinical benefits 12 months after intervention and relate those benefits to a loss of airway innervation. A more complete "denervation" is achieved during lung transplant, and yet, while patients acutely lose their cough reflex following lung transplant and there is an accompanying decrease in airway innervation, by 12 months cough responsiveness is restored and at 33 months (earlier time points were not analyzed) nerve fibers in the mucosa are again present (Duarte et al., Chest. 2008; 134(2):310-316). Do the authors anticipate the need for additional thermoplasty as nerve fibers re-innervate the distal airways?

Response: this is a very interesting point. At 12 months we found a trend to re-innervation, but without clinical deterioration. In large trials clinical improvement persisted at 5 years (see reference 20), and clinical data are the real target for the treatment rather than a possible re-innervation. Anyway this would be an intriguing aspect to clarify from a biological and pathophysiological point of view. A recent european consensus panel of experts on BT discussed this possibility without an agreement (article submitted for publication).