



## N-Acetylcysteine protects human bronchi by modulating the release of neurokinin A in an ex vivo model of COPD exacerbation

Luigino Calzetta<sup>a,\*</sup>, Paola Rogliani<sup>a</sup>, Francesco Facciolo<sup>b</sup>, Barbara Rinaldi<sup>c</sup>, Mario Cazzola<sup>a</sup>, Maria Gabriella Matera<sup>c</sup>

<sup>a</sup> Department of Experimental Medicine and Surgery, University of Rome “Tor Vergata”, Rome, Italy

<sup>b</sup> Regina Elena National Cancer Institute, Thoracic Surgery Unit, Rome, Italy

<sup>c</sup> Department of Experimental Medicine, Unit of Pharmacology, Second University of Naples, Naples, Italy



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### ABSTRACT

**Aims:** N-Acetylcysteine (NAC) reduces the risk of exacerbation of chronic obstructive pulmonary disease (COPD). Although NAC also has anti-inflammatory activity, the detailed mechanism leading to its protective role remains to be elucidated. We tested the impact of NAC against the effects of lipopolysaccharide (LPS) in an ex vivo model of COPD exacerbation, and investigated the role of neurokinin A (NKA) in this context.

**Main methods:** Isolated airways from COPD patients were incubated overnight with LPS (100 ng/ml). NAC was tested at concentrations resembling the plasma levels elicited by oral administration of NAC at 200 mg/day (very low dose), 600 mg/day (low dose) and 1.200 mg/day (high dose).

**Key findings:** NAC at high concentrations normalized the peroxidase activity, H<sub>2</sub>O<sub>2</sub>, malondialdehyde (MDA), nitric oxide, glutathione (GSH), total antioxidant capacity (TAC), and interleukin 6 (IL-6) (overall change 34.32% ± 4.22%, P < 0.05 vs. LPS-treated). NAC at low concentrations modulated peroxidase activity, H<sub>2</sub>O<sub>2</sub>, MDA, GSH, TAC, and IL-6 (overall change 34.88% ± 7.39%, P < 0.05 vs. LPS-treated). NAC at very-low concentrations was effective on peroxidase activity, H<sub>2</sub>O<sub>2</sub>, GSH, and IL-6 (overall change 35.05 ± 7.71%, P < 0.05 vs. LPS-treated). Binary logistic regression analysis indicated that the modulatory effect of NAC on NKA levels was associated with a reduction of pro-oxidant factors and IL-6, and selectively blocking the NK<sub>2</sub> receptor abolished such an association.

**Significance:** This study demonstrates that, along with its well-known antioxidant activity, the protective effect of NAC against the detrimental effect of LPS is due to the modulation of NKA and IL-6 levels.

### 1. Introduction

Oxidative stress is recognized to be a predisposing factor in the pathogenesis and development of chronic obstructive pulmonary disease (COPD). Consequently, targeting oxidative stress is likely to be beneficial as a treatment in COPD [1].

Glutathione (GSH), a carrier of an active thiol group in the form of a cysteine (Cys) residue, is an antioxidant agent that interacts with reactive oxygen species (ROS) [1]. GSH concentrations in bronchoalveolar lavage fluid (BALF) are reduced during COPD exacerbation compared with those detectable in stable COPD patients [2]. The loss of antioxidant capacity in the course of oxidative stress condition induces depletion in GSH and/or its precursor Cys. Unfortunately, Cys cannot be used as a GSH precursor because it is toxic at high concentrations, and because of its rapid metabolism and oxidation [3,4]. Maintaining

adequate levels of GSH is essential to counteract excessive ROS production during acute exacerbation of COPD (AECOPD) [1]. In this regard N-Acetylcysteine (NAC), an antioxidant and mucolytic agent that is a thiol-containing compound and serves as the substrate Cys in the synthesis of GSH by delivering sulfhydryl moieties, is effective in restoring the pool of intracellular GSH depleted during AECOPD [5].

Large randomized clinical trials and exhaustive meta-analyses showed that NAC is effective in reducing the risk of AECOPD [6–9]. This effect is likely due to ability of NAC to modulate the oxidative stress [10]. However, although the antioxidant properties of NAC are currently well known and include both direct (the free sulfhydryl group acts as source of reducing equivalents) and indirect (by restoring the intracellular GSH levels) effects [1], the mechanism of action leading to the anti-inflammatory activity of NAC still remains to be fully elucidated.

\* Corresponding author at: Department of Experimental Medicine and Surgery, University of Rome “Tor Vergata”, Via Montpellier 1, 00133, Rome, Italy.

E-mail addresses: [luigino.calzetta@uniroma2.it](mailto:luigino.calzetta@uniroma2.it) (L. Calzetta), [paola.rogliani@uniroma2.it](mailto:paola.rogliani@uniroma2.it) (P. Rogliani), [francesco.facciolo@ifo.gov.it](mailto:francesco.facciolo@ifo.gov.it) (F. Facciolo), [barbara.rinaldi@unicampania.it](mailto:barbara.rinaldi@unicampania.it) (B. Rinaldi), [mario.cazzola@uniroma2.it](mailto:mario.cazzola@uniroma2.it) (M. Cazzola), [mariagabriella.matera@unicampania.it](mailto:mariagabriella.matera@unicampania.it) (M.G. Matera).

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Lipopolysaccharide (LPS) is a major pro-inflammatory glycolipid component of the outer cell membrane of Gram-negative bacteria associated with many types of lung diseases, including COPD [11]. LPS inhalation induces increased neutrophils, macrophages and cytokines in sputum and bronchoalveolar lavage fluid (BALF), and similar inflammatory changes are observed during AECOPD [12]. Intriguingly, it has recently been proved that NAC elicits both antioxidant and anti-inflammatory effects in a concentration-dependent manner in human isolated bronchi of patients with COPD stimulated by LPS [12].

Since the challenge of airways with LPS induces ROS formation and neurogenic inflammation leading to the release of neurokinin A (NKA) [12,13], we have hypothesized that this tachykinin may, at least partially, trigger the oxidative stress and inflammation during AECOPD. Therefore, we tested in a validated ex vivo model of AECOPD the protective role of NAC against the detrimental effects induced by LPS in human isolated airways, and assessed whether the effect of NAC might be related to the modulation of NKA release and consequent activation of its specific receptor NK<sub>2</sub> [12]. Furthermore, we tested NAC at concentrations that reproduced ex vivo the plasma levels elicited by oral administration of NAC at 200 mg/day (very low dose), 600 mg/day (low dose) and 1.200 mg/day (high dose).

## 2. Materials and methods

### 2.1. Ethical approval and informed consent

Ethical approval (RS 60.15, 2015 Independent Ethical Committee, Fondazione PTV Policlinico Tor Vergata) and informed consent were consistent with the 2009 National Committee of Bioethics, National Committee of Bio-safety, Biotechnology and Sciences (Italy) recommendations on the collection of biological samples for research purposes, the 2010 Italian ethical and legal recommendations concerning the biobank and the research biorepository (Istituto Nazionale dei Tumori – Independent Ethics Committee, 2010), and the Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze per la Vita (Raccolta di campioni biologici a fini di ricerca, consenso informato, 2009; available at: [http://www.governo.it/bioetica/gruppo\\_misto/Consenso\\_Informato\\_allegato\\_Petrini\\_2009.pdf](http://www.governo.it/bioetica/gruppo_misto/Consenso_Informato_allegato_Petrini_2009.pdf)).

### 2.2. Tissue collection and preparation

Macroscopically normal airways were obtained from 10 moderate-to-severe COPD patients (6 male, 4 female, age 63.5 ± 3.6 years old) undergoing surgery for lung cancer. Samples were taken from an area as far as possible from the malignancy. Tissues were placed in Krebs-Henseleit (KH) buffer solution (NaCl, 119.0 mmol; KCl, 5.4 mmol; CaCl<sub>2</sub>, 2.5 mmol; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol; MgSO<sub>4</sub>, 1.2 mmol; NaHCO<sub>3</sub>, 25.0 mmol; glucose, 11.7 mmol; pH 7.4) containing indomethacin (5 μM) and transported to the Laboratory of Respiratory Clinical Pharmacology at the University of Rome Tor Vergata (Italy) from a nearby hospital.

In our laboratory, the airways were cut into rings (thickness 1–2 mm; diameter 4–6 mm) and transferred into a 10-ml High Tech 8 Channels Manual Compact Organ Bath system (Panlab Harvard Apparatus, Spain) containing KH-buffer (37 °C) and aerated with O<sub>2</sub>/CO<sub>2</sub> (95:5%). Tissues were allowed to equilibrate and the KH buffer was constantly changed.

### 2.3. Preparation of drugs

The following compounds were used: indomethacin (Sigma-Aldrich, Milan, Italy), GR159897 (Santa Cruz Biotechnology, Texas, US), LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, Milan, Italy), and NAC (Zambon, Milan, Italy). LPS and NAC were dissolved in distilled water, GR159897 was dissolved in dimethyl sulfoxide (DMSO), and indomethacin was first dissolved in ethanol and then diluted in the KH

buffer. The maximal final concentrations of DMSO (0.1%) and ethanol (0.02%) achieved in the organ bath did not influence the response of isolated tissues, as previously reported [13,14]. Appropriate dilutions were obtained in freshly prepared medium and stock solutions were stored at –80 °C until use. NAC dilutions were prepared daily before the experiments.

### 2.4. COPD exacerbation model

Bronchial rings were incubated overnight with KH buffer solution (negative control) or LPS (100 ng/ml, positive control) in order to mimic ex vivo the condition of airways during AECOPD in vivo [15–17].

Some LPS-incubated tissues were pre-treated with increasing concentrations of NAC ranging from 10 nM to 1 mM. Plasma concentrations of NAC reach values of ≈5 μM after oral administration of 200 mg/day, ≈16 μM after oral administration of 600 mg/day, and ≈35 μM after an oral administration of 1200 mg/day [12]. Therefore, in order to reproduce ex vivo the plasma bioavailability following very low, low and high oral doses of NAC, we specifically included NAC at 5 μM, 16 μM, and 35 μM in the range of concentrations tested in the bats.

### 2.5. Pro/antioxidant factors and inflammatory profile

The supernatants from all the treatments were collected in order to assess the impact of NAC on the pro/antioxidant response and on the inflammatory profile of human airways.

The pro-oxidant response was assessed by quantifying the activity of peroxidase and the concentrations of hydrogen peroxide, malondialdehyde (MDA), and nitric oxide. The protective response to oxidative stress was investigated by measuring the total antioxidant capacity (TAC), GSH, and the superoxide dismutase (SOD) activity. The levels of cytokines, namely interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor alpha (TNF-α) were also determined.

The quantification of pro/antioxidant factors and cytokines was performed by using colorimetric, fluorometric and ELISA assays characterized by high sensitive detection limits and high specificity, in accordance with the manufacturers' datasheets (BioVision, Milpitas, CA, USA; B-Bridge International, Santa Clara, CA, USA; Epigentek Group Inc, Farmingdale, NY, USA; Assaypro, St. Charles, MO, USA; RayBiotech, Norcross, GA, USA). Detailed information on the quantification of pro/antioxidant factors and cytokines are available in the Supplemental file.

### 2.6. Neurokinin A quantification and NK<sub>2</sub> receptor blockage

In order to ascertain whether the protective effect of NAC in LPS-incubated bronchi was related to the release of NKA followed by the activation of its specific receptor, we have quantified the levels of NKA and selectively blocked the NK<sub>2</sub> receptor.

Thus, the supernatants of isolated organ bath system were also used to quantify the levels of NKA released in response to LPS incubation and NAC treatment. The quantification of NKA was performed by an ELISA assay in accordance with manufacturers' datasheets (RayBiotech, Norcross, GA, USA). Detailed information on the quantification of NKA are available in the Supplemental file.

Moreover, some experiments were carried out in the presence of the selective NK<sub>2</sub> receptor antagonist GR159897 administered at 300 nM (pK<sub>b</sub>: 8.57) [18].

The final concentration in the baths of the NK<sub>2</sub> receptor antagonist was two logarithms greater than their pK<sub>b</sub> value in order to selectively antagonize the targeted receptor [13].

### 2.7. Data analysis

The concentration-response curve of NAC with regard to NKA levels

was reported by using an appropriate non-linear regression fitting model, as previously reported by Motulsky and Christopoulos [19], in order to calculate the maximal effect ( $E_{max}$ ), the concentration inducing 50%  $E_{max}$  ( $EC_{50}$ ), and the potency ( $pEC_{50}$ ), where  $pEC_{50} = -\log EC_{50}$ .

Bubble charts were used to display three dimensions of data, with each triplet ( $v_1, v_2, v_3$ ) represented by the concentrations of NAC ( $v_1$ ) and NKA ( $v_2$ ) on the x and y axes, respectively, and the size of disk ( $v_3$ ) reporting the changes (fold) vs. control of pro/antioxidant factors and cytokines. Since the human visual system naturally experiences the size of a disk in terms of its area, the data for  $v_3$  are reported as area, rather than radius or diameter [20].

Linear regression analysis was carried out to assess the level of association between the impact of NAC on NKA release and the overall changes in the levels of pro/antioxidant factors and cytokines. The causal relationship between the impact of NAC on NKA levels and the concentrations of pro/antioxidant factors and cytokines was investigated through binary logistic regression by assessing the applying the goodness of fit via the Hosmer–Lemeshow test, the increase in correctness of classification of the regression models, and the regression coefficients.

Experiments were performed on  $n = 5$  bronchi collected from different patients and values are presented as mean  $\pm$  SEM. According to the availability and vitality of the tissues, each single treatment was carried out by using specimens collected from the same patient, and experiments were repeated 5 times in samples originating from 5 different donors. When the amount of the sample from one patient was not sufficient for all the treatments, the remaining treatments were carried out on specimens collected from other patients in parallel with further positive and negative controls.

Evaluation of the pro/antioxidant activity and quantification of cytokines were normalized for 100 mg of bronchial tissue and carried out by performing the experiments in triplicate.

Statistical significance was assessed by two-way analysis of variance (ANOVA) and the level of statistical significance was defined as  $P < 0.05$ . Data analysis was performed by using Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA) and bubble charts were produced using Microsoft Excel 2008 for MAC.

### 3. Results

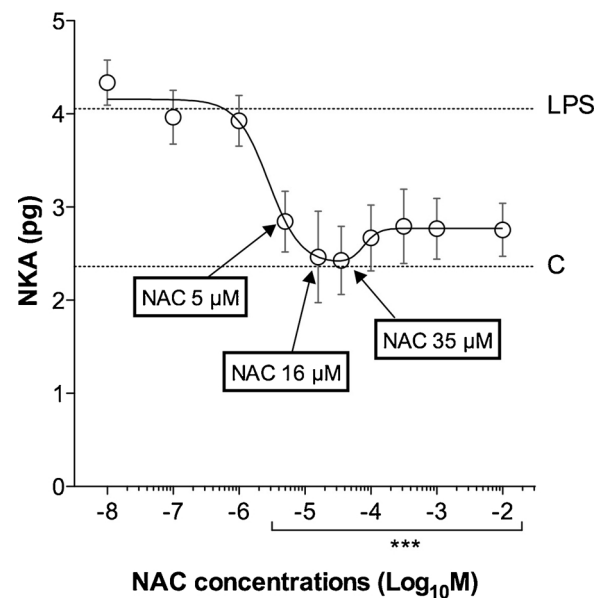
#### 3.1. Impact of NAC on the levels of NKA in human isolated bronchi incubated with LPS

The overnight challenge with LPS (100 ng/ml) elicited a significant ( $P < 0.05$ ) increase in NKA concentrations compared with controls ( $4.05 \pm 0.17$  pg and  $2.36 \pm 0.57$  pg, respectively).

NAC administered from 10 nM to 1  $\mu$ M did not prevent the release of NKA ( $P > 0.05$  vs LPS-incubated bronchi), whereas concentrations  $\geq 5$   $\mu$ M significantly inhibited ( $P < 0.001$ ) the levels of NKA.

The best fit equation that described the impact of NAC on the release of NKA was the bell-shaped concentration-response curve described by the following model:  $\text{Span1} = 2.77\text{--}4.54$ ;  $\text{Span2} = 4.16\text{--}4.54$ ;  $\text{Section1} = \text{Span1}/(1 + 10^{((-5.55-X)*1.80)})$ ;  $\text{Section2} = \text{Span2}/(1 + 10^{((X+4.11)*3.92)})$ ;  $Y = 4.54 + \text{Section1} + \text{Section2}$ .

This model indicated that the  $pD_2$  of NAC was  $5.55 \pm 0.32$ , and that  $E_{max}$  was reached for NAC administered at 35  $\mu$ M, when the concentrations of NKA ( $2.43 \pm 0.57$  pg) were not significantly ( $P > 0.05$ ) different compared with those detected in control bronchi. The bell-shaped equation implies that the effectiveness of NAC was numerically reduced when administered at concentrations higher than  $> 35$   $\mu$ M (NKA plateau2:  $2.77 \pm 0.19$  pg). Details on the bell-shaped concentration-response curve induced by NAC with regard to the NKA concentrations are reported in Fig. 1.



**Fig. 1.** Bell-shaped concentration-response curve elicited by NAC with regard to the release of NKA in human isolated bronchi stimulated overnight with LPS (100 ng/ml). Points represent the mean  $\pm$  SEM of experiments performed in  $n = 5$  different samples. Experiments were undertaken in triplicate in order to ensure the reliability of single values.  $***P < 0.001$  vs. LPS-incubated bronchi (statistical significance assessed by two-way ANOVA). C: control; LPS: lipopolysaccharide; NAC: N-acetylcysteine; NKA: neurokinin A.

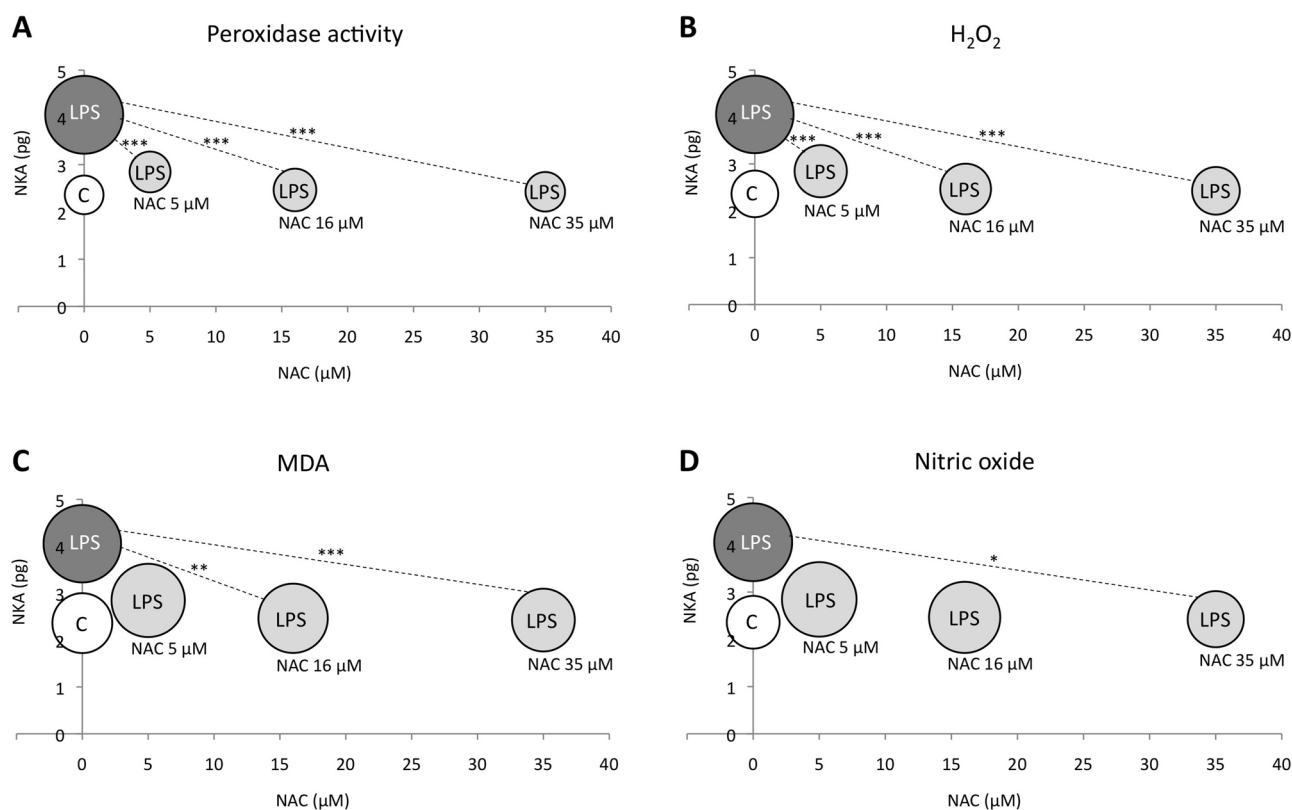
#### 3.2. Impact of bioavailable concentrations of NAC and relative NKA release on the oxidative stress and inflammatory profile of human isolated bronchi in response to LPS

Along with increased levels of NKA, the overnight incubation with LPS (100 ng/ml) induced a significant pro-oxidant and pro-inflammatory response of human airways ( $1.61 \pm 0.12$  fold and  $10.56 \pm 1.57$  fold vs. control, respectively;  $P < 0.001$ ), as well as a significant reduction of the antioxidant capacity (overall:  $0.69 \pm 0.08$  fold vs. control;  $P < 0.001$ ).

The combined analysis of the impact of the bioavailable concentrations of NAC (16–35  $\mu$ M) in plasma and the relative NKA levels with regard to the oxidative profile elicited by the challenge with LPS indicated that NAC normalized the NKA concentrations. Furthermore, NAC administered from 5  $\mu$ M to 35  $\mu$ M was effective in preventing an increase in peroxidase activity and  $H_2O_2$  release ( $0.53 \pm 0.05$  fold and  $0.65 \pm 0.03$  fold vs. LPS, respectively;  $P < 0.001$ ). Although the increase in MDA was prevented by NAC administered at 16–35  $\mu$ M ( $0.85 \pm 0.01$  fold vs. LPS;  $P < 0.01$ ), only NAC administered at the highest concentration (35  $\mu$ M) was effective in inhibiting the release of nitric oxide ( $0.72 \pm 0.04$  fold vs. LPS;  $P < 0.05$ ) (Fig. 2).

The effect of NAC administered from 5  $\mu$ M to 35  $\mu$ M on the NKA levels was also significantly associated with increased concentrations of GSH ( $1.58 \pm 0.09$  fold vs. LPS;  $P < 0.05$ ), whereas only NAC administered at 16–35  $\mu$ M normalized the levels of TAC ( $1.24 \pm 0.12$  fold vs. LPS;  $P < 0.05$ ). NAC, and the relative modulation of NKA, had no significant effect on SOD activity ( $1.15 \pm 0.20$  fold vs. LPS;  $P > 0.05$ ) (Fig. 3).

Although the modulatory effect of NAC administered from 5  $\mu$ M to 35  $\mu$ M on NKA levels had a beneficial impact against the IL-6 increase induced by LPS challenge ( $0.66 \pm 0.03$  fold vs. LPS;  $P < 0.001$ ), no effect was detected with regard to the concentrations of IL-1 $\beta$ , IL-8 and TNF- $\alpha$  (overall:  $1.01 \pm 0.05$  fold vs. LPS;  $P > 0.05$ ) (Fig. 4).



**Fig. 2.** Combined analysis of the impact of the bioavailable plasma concentrations of NAC (16–35 µM) and the relative release of NKA with regard to the pro-oxidant response elicited by the overnight incubation with LPS (100 ng/ml). The area of the bubbles was determined by the values of peroxidase activity (A), H<sub>2</sub>O<sub>2</sub> (B), MDA (C), and nitric oxide (D), and are expressed as fold vs. control. Points represent the means of experiments performed on n = 5 different samples. Experiments were undertaken in triplicate in order to ensure the reliability of single values. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 vs. LPS-incubated bronchi (statistical significance assessed by two-way ANOVA). C: control; LPS: lipopolysaccharide; MDA: malondialdehyde; NAC: N-acetylcysteine; NKA: neurokinin A.

### 3.3. Regression analysis

The linear regression analysis identified a significant association between the beneficial impact of NAC against NKA release with regard to the overall changes in antioxidant factors (model summary:  $R^2 = 0.98$ , SE of estimates 0.16,  $P < 0.01$ ) and pro-oxidant factors (model summary:  $R^2 = 0.79$ , SE of estimates 0.42,  $P < 0.05$ ), whereas a signal of association was detected with regard to the modulation of the inflammatory profile (model summary:  $R^2 = 0.76$ , SE of estimates 0.50,  $P = 0.14$ ) (Fig. 5A–C).

In order to adequately describe the relationship between the impact of NAC on NKA levels with regard to the pro/antioxidant factors and pro-inflammatory cytokines, we have carried out a binary logistic regression analysis, by assuming as effective the concentrations of NAC that, after the challenge with LPS (100 ng/ml), reduced the concentrations of NKA to levels that were not significantly ( $P > 0.05$ ) different compared with controls. The regression models indicated that the modulation of NKA induced by NAC was significantly (overall  $P < 0.05$ ) associated with the reduction of all the pro-oxidant factors investigated in this study. A signal (overall  $P \approx 0.1$ ) of association was detected for the increase in TAC and GSH, although the latter was characterized by a weak goodness of fit. Concerning the levels of cytokines, the modulatory effect of NAC on NKA levels was significantly ( $P < 0.05$ ) associated with IL-6, but not with IL-1 $\beta$ , IL-8 and TNF- $\alpha$ .

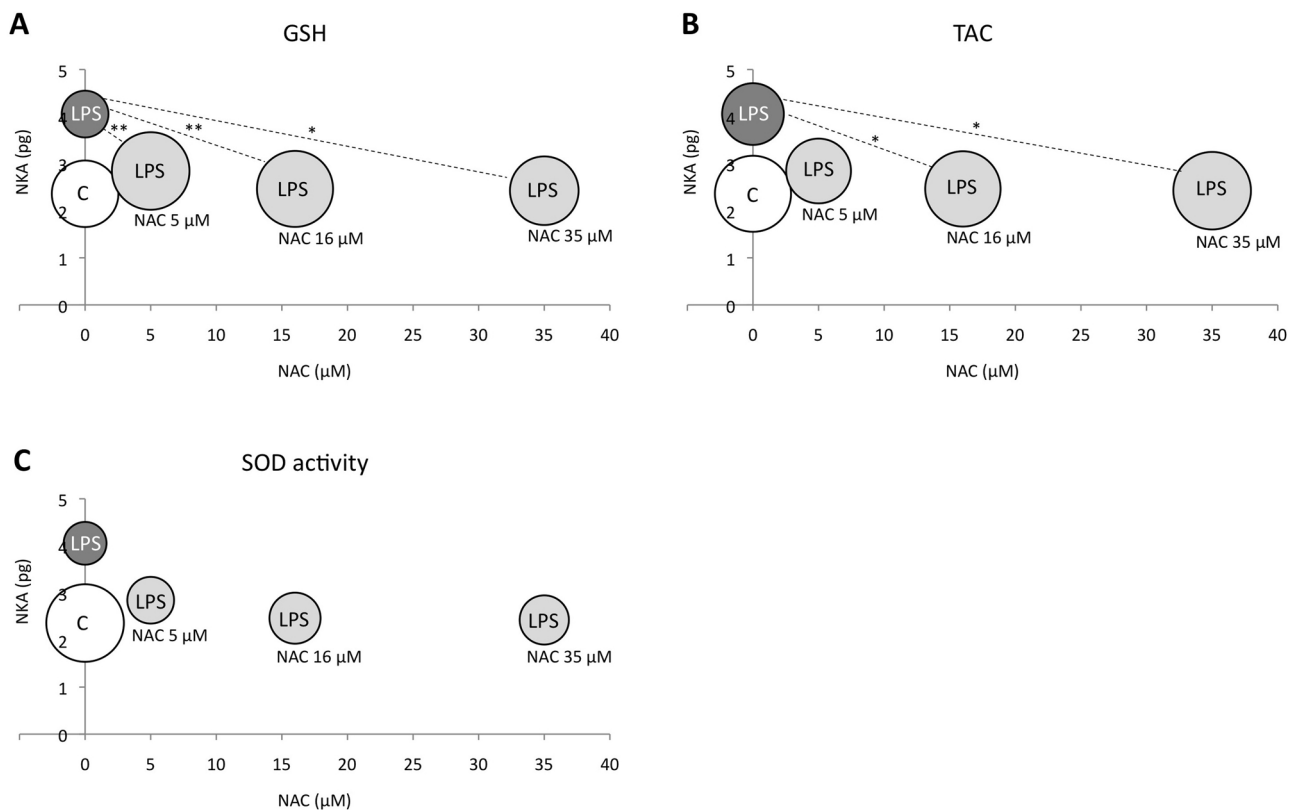
The presence in the baths of the selective NK<sub>2</sub> receptor antagonist GR159897 administered at 300 nM abolished the protective association between NAC and NKA release with regard to the levels of pro-oxidant factors (peroxidase activity, H<sub>2</sub>O<sub>2</sub>, MDA, and NO) and IL-6 release in LPS-incubated airways (overall logistic regression significance:  $P > 0.05$ ). More details on the logistic regression output are reported in Table 1.

### 4. Discussion

The results of this study demonstrate that NAC elicits both anti-oxidant and anti-inflammatory effects in human airways in an ex vivo model of AECOPD. In particular, NAC at high concentrations (35 µM, corresponding to the plasma level after oral administration of NAC 1200 mg/day) normalized the peroxidase activity and the levels of H<sub>2</sub>O<sub>2</sub>, MDA, nitric oxide, GSH, TAC, and IL-6 after an overnight challenge with LPS. NAC at low concentrations (16 µM, corresponding to the plasma level after oral administration of NAC 600 mg/day) did not modulate the levels of nitric oxide. NAC at very low concentrations (5 µM, corresponding to the plasma level after oral administration of NAC 200 mg/day) was not effective on the levels of MDA, nitric oxide or TAC.

Our findings also confirm previous data on the beneficial effect of NAC against the increased levels of NKA induced by LPS stimulation [12]. However, in the current study we have investigated the impact of concentrations of NAC that mimic plasma concentration following its oral administration. Surprisingly, we found that the best fit equation that describes the impact of NAC on the levels of NKA was a bell-shaped concentration-response curve, in which the inhibitory response at low-medium concentrations was greater than that elicited at higher concentrations. This model is more complicated than the standard monotonic sigmoid concentration-response curve, however in the present study we have evaluated a sufficient number of data points to adequately define both phases of the response.

Drugs reported to both activate and inhibit transmembrane receptors, and the complicated stimulus-response relationships caused by the promiscuity of different receptors, offer the classic mechanisms through which bell-shaped curves may be understood [21,22]. Unfortunately, since NAC does not interact with transmembrane receptors,



**Fig. 3.** Combined analysis of the impact of the bioavailable plasma concentrations of NAC (16–35 µM) and the relative release of NKA with regard to the antioxidant response elicited by the overnight incubation with LPS (100 ng/ml). The area of the bubbles was determined by the values of GSH (A), TAC (B), and SOD activity (C), and are expressed as fold vs. control. Points represent the means of experiments performed on  $n = 5$  different samples. Experiments were undertaken in triplicate in order to ensure the reliability of single values. \* $P < 0.05$  and \*\* $P < 0.01$  vs. LPS-incubated bronchi (statistical significance assessed by two-way ANOVA). C: control; LPS: lipopolysaccharide; GSH: glutathione; NAC: N-acetylcysteine; NKA: neurokinin A; SOD: superoxide dismutase; TAC: total antioxidant capacity.

but acts as a precursor for the substrate Cys in the synthesis of GSH [5], the cause of the unusual bell-shaped concentration–response curve reported in this study remains to be elucidated. The peculiar fitting model induced by NAC with regard to the levels of NKA may explain why conflicting *in vitro* and *in vivo* dose-effect findings have been reported, especially with regard to the anti-inflammatory activity of this antioxidant agent [10]. The pharmacological characterization of NAC that we provided in this study undoubtedly indicates that the  $E_{max}$  corresponds to the plasma levels induced by the oral administration of NAC at 1200 mg/day, and that higher should not improve the protection against NKA release, at least during LPS challenge in isolated airways of COPD patients.

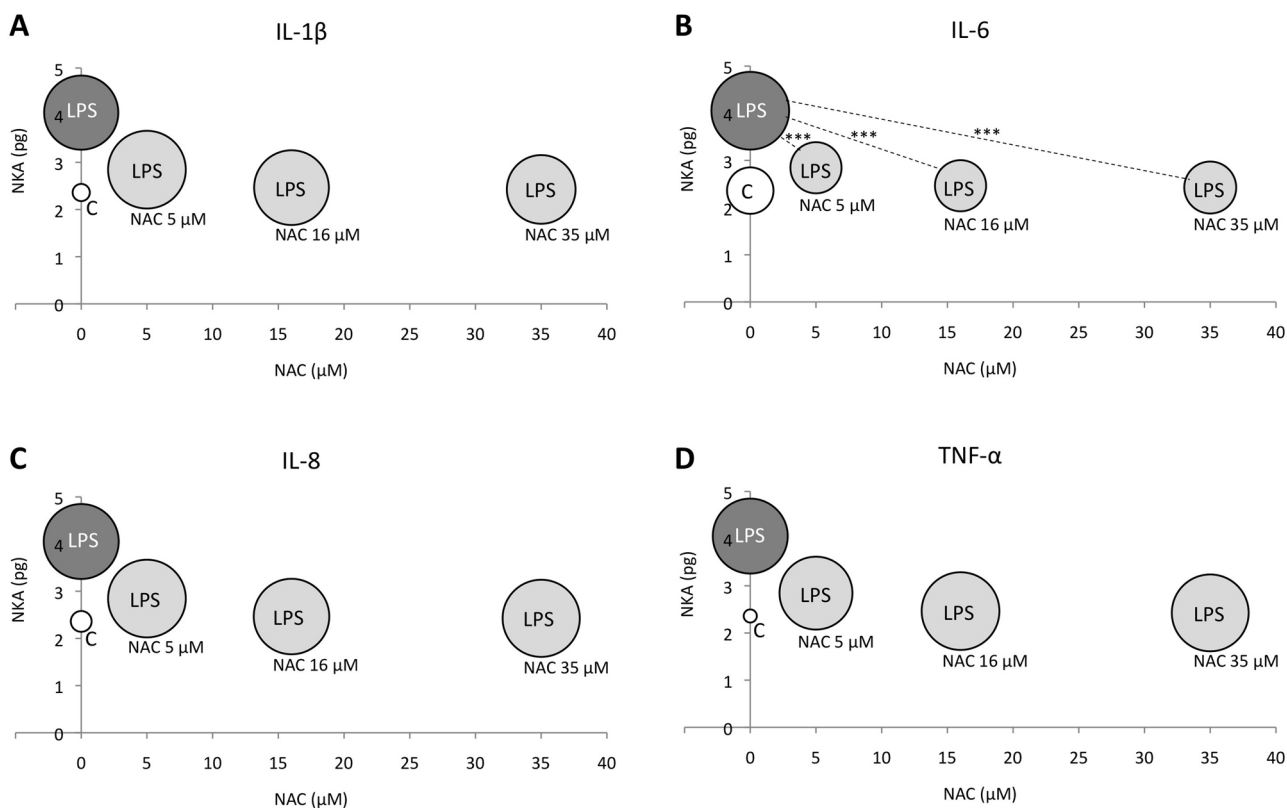
Intriguingly, this preclinical finding is consistent with the results of clinical trials that have demonstrated that very high doses of NAC administered at 1800 mg/day for 8 weeks did not provide any clinical benefit in patients with COPD and chronic bronchitis [23], compared with longer treatments with NAC administered at 1200 mg/day [8,9]. Therefore, it seems that there is no pharmacological rationale for administering NAC at doses higher than 1200 mg/kg. Nevertheless, in some specific clinical conditions such as during oxygen therapy for COPD, the antioxidant activity of NAC may be more effective when administered at 1800 mg/day [24].

The linear regression analysis demonstrates an association between the beneficial impact of NAC against the NKA levels and the overall changes in the concentrations of pro/antioxidant factors. On the other hand, only a signal for an association was detected regarding the overall levels of pro-inflammatory cytokines. In order to better explain these relationships, and identify the potential mechanisms beyond the well-known antioxidant effect of NAC mediated by restoration of the depleted pool of intracellular GSH during AECOPD, we have also

performed a binary logistic regression analysis and used the selective  $NK_2$  receptor antagonist GR159897 in our experiments.

The binary logistic regression analysis indicates that the modulatory effect of NAC on NKA levels represents a possible mechanism leading to improved reduction of pro-oxidant factors, namely peroxidase activity,  $H_2O_2$ , MDA, and nitric oxide. As expected, the reduction of NKA does not seem to be responsible for the increased levels of GSH and SOD activity. However, we found a signal for an association between the reduction of NKA concentrations and the increase of TAC. This last finding may be explained by the fact that TAC is a non-specific assay that measures the overall capability to counteract ROS, and its modulation may be indirectly mediated by the reduction in the levels of pro-oxidant factors. Our analysis also indicates that the anti-inflammatory effect of NAC administered from very low to high concentrations specifically focused against the increase in IL-6, which is clearly mediated by the reduction of NKA concentrations.

Indeed, the significant association between the modulation of NKA by NAC and the reduction of all the pro-oxidant factors and IL-6 resulting from the logistic regression analysis does not represent a biological demonstration of a causality link between these events. However, since we have also demonstrated that GR159897 abolished the protective association between NAC and NKA release with regard to oxidant/antioxidant imbalance and cytokine release, here we provide the experimental evidence that the modulation of NKA concentrations leading to the activation of  $NK_2$  receptor represents an important mechanism of action that allows NAC to improve both the antioxidant and anti-inflammatory status. Probably such a mechanism of action is effective to counteract the deleterious effects of AECOPD and LPS challenge. In particular, previous studies demonstrated that LPS might directly activate the toll-like receptor 4 (TLR4) in sensory neurons,



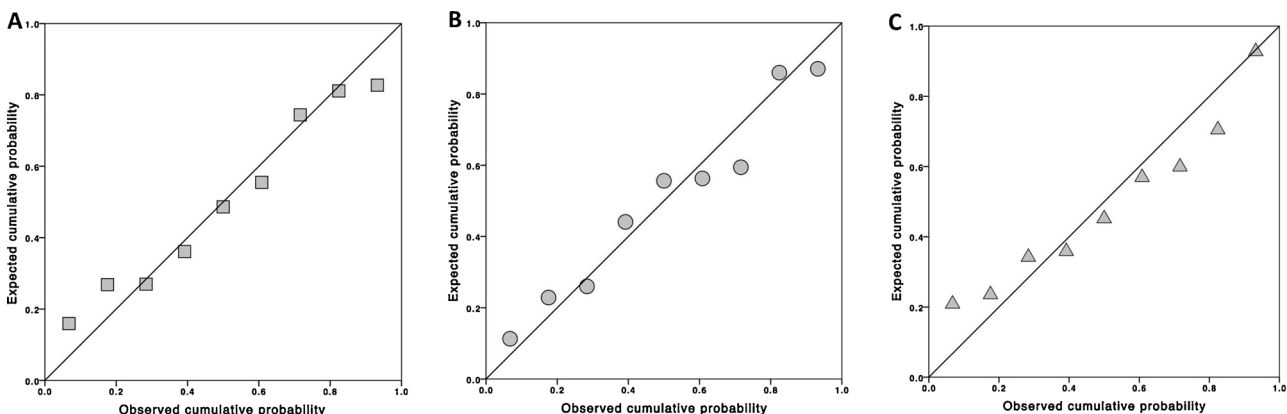
**Fig. 4.** Combined analysis of the impact of the bioavailable plasma concentrations of NAC (16–35 µM) and the relative release of NKA with regard to the pro-inflammatory response elicited by the overnight incubation with LPS (100 ng/ml). The area of the bubbles was determined by the values of IL-1β (A), IL-6 (B), IL-8 (C), and TNF-α (D), and are expressed as fold vs. control. Points represent the means of experiments performed on n = 5 different samples. Experiments were undertaken in triplicate in order to ensure the reliability of single values. \*\*\*P < 0.001 vs. LPS-incubated bronchi (statistical significance assessed by two-way ANOVA). C: control; IL: interleukin; LPS: lipopolysaccharide; NAC: N-acetylcysteine; NKA: neurokinin A; TNF- α: tumour necrosis factor alpha.

leading to sensitization/overexpression of transient receptor potential vanilloid type 1 (TRPV1), which in turn, induces an increased release of NKA [13,25]. Considering that even low concentrations of NKA may facilitate the parasympathetic pathway driven by the vagus nerve [14], this neuropeptide seems to play a pivotal role in fine-tuning neurogenic inflammation by inducing airway inflammation, bronchoconstriction, mucus secretion, and plasma exudation [26]. Pro-inflammatory neuropeptides, including NKA, are transported antidromically via sensory nerves back to the peripheral endings and locally released to propagate the events of neurogenic inflammation [26].

The challenge with LPS increases the levels of NKA in the airways [13,14,16], and we cannot exclude that IL-6 may itself elicit a further

increase in NKA release. It has been demonstrated that IL-6 increases the release of NKA in the hypothalamic-pituitary axis [27]. Furthermore, although we cannot determine whether the neurogenic inflammation elicited by LPS challenge induces oxidative stress by itself or vice versa, it is evident that acting on NKA levels by administering NAC has a beneficial impact on both the antioxidant and anti-inflammatory profile of human isolated airways.

Certainly, we cannot omit that targeting neurokinin receptors failed to show any clinical benefit in humans with asthma [28–31], but to date no clinical trials have been carried out by specifically blocking NK<sub>2</sub> receptor in COPD patients. In any case, in an animal model of COPD the dual NK<sub>1</sub> and NK<sub>2</sub> receptor antagonist DNK333 was able to block the



**Fig. 5.** Linear regression analysis via normal P-P plot of the modulatory effect of NAC and the relative release of NKA with regard to the levels of pro-oxidant factors (A), antioxidant factors (B) and pro-inflammatory cytokines (C). NAC: N-acetylcysteine; NKA: neurokinin A.

**Table 1**

Logistic regression output describing the relationship between the impact of NAC on NKA levels and the pro/antioxidant factors and pro-inflammatory cytokines in human airways stimulated by LPS (100 ng/ml).

	Significance (P)	Goodness of fit (P) <sup>a</sup>	Increase in correctness of classification	Regression coefficient
Peroxidase activity	*	≈1	33.33	−32.11
H <sub>2</sub> O <sub>2</sub>	**	≈1	33.33	−8.10
MDA	*	≈1	33.33	−523.97
NO	*	≈1	33.33	−71.13
GSH	0.109	0.568	11.1	8.18
TAC	0.088	≈1	33.33	4.34
SOD activity	NS	0.414	0	0.40
IL-1β	NS	0.254	0	0.06
IL-6	*	0.860	33.33	−810.60
IL-8	NS	0.591	0	−14.33
TNF-α	NS	0.436	0	−16.32

IL: interleukin; LPS: lipopolysaccharide; MDA: malondialdehyde; NAC: N-acetylcysteine; NKA: neurokinin A; SOD: superoxide dismutase; TAC: total antioxidant capacity; TNF-α: tumour necrosis factor alpha. NS: not significant (P ≥ 0.05).

\* P < 0.05.

\*\* P < 0.01.

<sup>a</sup> Via Hosmer–Lemeshow test (the higher the P value, the better the model).

mucus secretion in the airways assessed in vivo and non-invasively by magnetic resonance imaging [32].

Sadowska and colleagues [10] suggested that the concentrations of NAC necessary to reduce inflammation are greater than those necessary to elicit an anti-oxidant effect. We recently supported this hypothesis [12], because we demonstrated that, over a wide range of concentrations reproducible exclusively in ex vivo conditions, the levels of NAC necessary to modulate the inflammatory response detectable in LPS-stimulated bronchi were at least one logarithm higher than those required to elicit antioxidant effects. Thus, we supposed that the anti-inflammatory effect of NAC could be secondary to its antioxidant activity. Nevertheless, the novel findings provided by this study definitely demonstrate that NAC elicits specific anti-inflammatory activity against IL-6 yet at low concentrations, corresponding to the oral administration of NAC at 200 mg/day. Intriguingly, the modulatory effect of NAC on the levels of IL-6 remained stable up to higher concentrations corresponding to the oral administration of NAC at 1200 mg/day.

Overall, the evidence that the beneficial impact of NAC is mediated by a proven antioxidant activity together with a specific anti-inflammatory effect against increase in IL-6 is of interest. It is widely recognized that IL-6 concentrations are increased in the exhaled breath condensate and sputum of COPD patients, and that the concentrations of IL-6 reflect the level of the airway inflammation [33,34]. Furthermore, AECOPD are accompanied by enhancement of serum IL-6 levels and plasma fibrinogen [33,35]. The latter is the only validated biomarker for patients with COPD who are at high risk for exacerbations and/or all-cause mortality [36]. However IL-6, besides being a promising serum biomarker [37], seems to have a pivotal role in AECOPD, especially those characterized by coinfection of rhinovirus and the Gram-negative pathogen *Haemophilus influenzae* [37,38].

## 5. Conclusions

The results of this study suggest that NAC could have an effect in reducing the risk of AECOPD not only by modulating the oxidant/antioxidant profile in human airways, but also by eliciting an anti-inflammatory activity that is secondary to the reduction of NKA concentrations. The greater effectiveness of NAC administered at high doses vs. low doses in terms of protection against the risk of AECOPD may be explained not only by an increase in the total daily dose of this

medication, but also by the more stable plasma levels elicited by NAC 600 mg twice daily (total daily dose 1200 mg) compared with NAC 600 mg once daily (total daily dose 600 mg). Finally, the findings of this research further support the evidence that NAC may modulate the vicious circle between oxidative stress and neurogenic inflammation, a deleterious condition that characterizes the airways of patients chronically colonized by Gram-negative bacteria and experiencing AECOPD [12].

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## Authors' contributions

LC: conducted experiments, acquired data, designed the research study, wrote the manuscript. PR: contributed essential reagents and tools, reviewed the manuscript. FF: conducted experiments. BR: reviewed the manuscript. MC: designed the research study, contributed essential reagents and tools, wrote the manuscript. MGM: designed the research study, reviewed the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

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Not applicable.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2018.04.011>.

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