

Effects of N-acetyl-L-cysteine on the membrane vesicle release and growth of respiratory pathogens

Citation for published version (APA):

Volgers, C., Benedikter, B. J., Grauls, G. E., Hellebrand, P. H. M., Savelkoul, P. H. M., & Stassen, F. R. M. (2017). Effects of N-acetyl-L-cysteine on the membrane vesicle release and growth of respiratory pathogens. *Fems Microbiology Letters*, 364(9), [087]. <https://doi.org/10.1093/femsle/fnx087>

Document status and date:

Published: 01/05/2017

DOI:

[10.1093/femsle/fnx087](https://doi.org/10.1093/femsle/fnx087)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

Taverne

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

RESEARCH LETTER – Pathogens & Pathogenicity

Effects of N-acetyl-L-cysteine on the membrane vesicle release and growth of respiratory pathogens

Charlotte Volgers¹, Birke J. Benedikter¹, Gert E. Grauls¹, Pauline H. M. Hellebrand¹, Paul H. M. Savelkoul^{1,2} and Frank R. M. Stassen^{1,*}†

¹School of Nutrition and Translational Research in Metabolism (NUTRIM), Department of Medical Microbiology, Maastricht University Medical Center⁺, 6229 HX Maastricht, The Netherlands and ²Department of Medical Microbiology and Infection Control, VU University Medical Center, 1081 BT Amsterdam, The Netherlands

*Corresponding author: School of Nutrition and Translational Research in Metabolism (NUTRIM), Department of Medical Microbiology, Maastricht University Medical Center⁺, P. Debyealaan 25, 6229 HX Maastricht, The Netherlands. Tel: +31-43-3874663; Fax: +31-43-3876643; E-mail: f.stassen@maastrichtuniversity.nl

One sentence summary: N-acetyl-L-cysteine enhances the membrane vesicle release but inhibits the bacterial growth and proinflammatory response to bacterial membrane vesicles of non-typeable *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*.

Editor: David Clarke

†Frank R.M. Stassen, <http://orcid.org/0000-0003-3710-0948>

ABSTRACT

Bacterial infections contribute to the disease progression of chronic obstructive pulmonary disease *e.g.* by stimulating mucus production in the airways. This increased mucus production and other symptoms are often alleviated when patients are treated with mucolytics such as N-acetyl-L-cysteine (NAC). Moreover, NAC has been suggested to inhibit bacterial growth. Bacteria can release membrane vesicles (MVs) in response to stress, and recent studies report a role for these proinflammatory MVs in the pathogenesis of airways disease. Yet, until now it is not clear whether NAC also affects the release of these MVs. This study set out to determine whether NAC, at concentrations reached during high-dose nebulization, affects bacterial growth and MV release of the respiratory pathogens non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis* (Mrc), *Streptococcus pneumoniae* (Spn) and *Pseudomonas aeruginosa* (Psa). We observed that NAC exerted a strong bacteriostatic effect, but also induced the release of proinflammatory MVs by NTHi, Mrc and Psa, but not by Spn. Interestingly, NAC also markedly blunted the release of TNF- α by naive macrophages in response to MVs. This suggests that the application of NAC by nebulization at a high dosage may be beneficial for patients with airway conditions associated with bacterial infections.

Keywords: membrane vesicles; N-acetyl-L-cysteine; non-typeable *Haemophilus influenzae*; *Moraxella catarrhalis*; *Streptococcus pneumoniae*; *Pseudomonas aeruginosa*

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a respiratory condition that poses a huge burden on the society worldwide (Pauwels 2001; Lopez *et al.* 2006). Patients with COPD of-

ten show an increased mucus production, sputum purulence and cough (Hauber, Foley and Hamid 2006; Miravittles 2011; Montes De Oca *et al.* 2012). To alleviate symptoms, mucolytics are frequently prescribed (van Overveld *et al.* 2005; Poole 2006;

Sadowska et al. 2006; Poole, Chong and Cates 2015). A commonly administered mucolytic is N-acetyl-L-cysteine (NAC): a small thiol-containing antioxidant that acts by disrupting disulphide bonds in mucus (Sheffner 1963; Poole 2006). Two recent meta-analyses concluded that patients with COPD experience less exacerbations after oral NAC administration (Cazzola et al. 2015; Poole, Chong and Cates 2015).

Bacterial respiratory infections are the main cause of exacerbations in patients with COPD and bacteria also have been shown to contribute the progression of stable disease (Sethi and Murphy 2008; Marin et al. 2012; King, MacDonald and Bardin 2013; Desai et al. 2014). A variety of studies have shown that NAC does not only have anti-oxidant or anti-inflammatory characteristics, but also bacteriostatic properties (Parry and Neu 1977; Riise et al. 2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004; Zhao and Liu 2010). These bacteriostatic effects of NAC are thought to be mediated by the inhibition of cysteine utilization by bacteria (Parry and Neu 1977). It is now understood that cysteine deprivation, besides its bacteriostatic action, also induces a bacterial stress response which is characterized by the release of bacterial membrane vesicles (MVs) (van de Waterbeemd et al. 2013). MVs carry several bacterial virulence factors causing them to exert strong proinflammatory responses (as reviewed in Brown et al. 2015; Schwechheimer and Kuehn 2015).

Therefore, in this study we examined the effect of NAC on both the MV release and growth of the common respiratory pathogens non-typeable *Haemophilus influenzae*, (NTHi), *Moraxella catarrhalis* (Mrc), *Streptococcus pneumoniae* (Spn) and *Pseudomonas aeruginosa* (Psa). Moreover, we determined if NAC affects the MV-induced proinflammatory responses.

MATERIALS AND METHODS

Reagents and antibodies

NAC (Sigma, St. Louis, MO, USA) was dissolved at 200 mM and the pH was corrected to 7.0–8.0. Antibodies against CD63 (unconjugated, mouse-anti-human clone H5C6) and CD81 (PE conjugated, mouse-anti-human clone JS-81) were obtained from BD (BD Bioscience, Franklin Lakes, NJ, USA). The anti-*Haemophilus influenzae* type b (α -Hib; clone 1079/457) monoclonal antibody was obtained from Acris (Acris GmbH, Herford, Germany). The rabbit serum against Mrc (strain A 1.39N, isolated from children in a primary school in Nieuwegein, the Netherlands (1989)), was kindly provided by Dr J. Hays (Erasmus University, Rotterdam, the Netherlands). The antibody against Psa was from Antibodies online (Aviva Systems Biology, San Diego, CA, USA, neat raised in guinea pig). Antibodies were purified using the antibody serum purification kit based on protein A (Abcam, Cambridge, MA, USA). For flow cytometric analysis, detection antibodies were PE conjugated using PE-labeling kits from Abcam. One hundred microgram of each antibody was PE conjugated according to the manufacturers' instructions.

Bacterial strains and culturing

The following bacterial strains were used: NTHi (ATCC-49247), Psa (ATCC-27853), Spn (ATCC-49619) and a Mrc clinical isolate (University Medical Centre Maastricht (MUMC+), the Netherlands). Mrc, Spn and Psa were pre-cultured on blood plates, and NTHi was cultured on vitalex-supplemented chocolate agar plates (Oxoid, Wesel, Germany) overnight at 5% CO₂ and 37°C. After overnight pre-culture, bacterial suspensions with a density of 0.5 McFarland (1.5×10^8 colony forming units (cfu)/ml) were

prepared. These suspensions were then used to prepare bacterial cultures or for infection experiments. Bacteria were cultured at 5×10^7 cfu/ml with or without NAC (5, 25 or 50 mM) for 6 h in 10 ml RPMI1640. NTHi and Mrc were cultured in medium supplied with 10 μ g/ml hemin and nicotinamide adenine dinucleotide (both from Sigma). After 6 h, cultures were centrifuged at $1200 \times g$ for 10 min, at room temperature. The pelleted bacteria were washed, diluted in phosphate-buffered saline (PBS) and the optical density was determined at 600 nm in optical methacrylate disposable cuvettes (Sarstedt, Newton, NC, USA). The supernatant was again centrifuged at $1200 \times g$ for 10 min to deplete possible remnant bacteria, followed by filtering the supernatants through 0.22 μ m filters. To allow MV quantification by tunable resistive pulse sensing, supernatants were concentrated 20 times to a final volume of 500 μ l by centrifugation at $4000 \times g$ for 15 min using Amicon ultra-15 10-kDa centrifugal filter units (Millipore, Billerica, MA, USA).

Bacterial MVs for the stimulation experiments were obtained from bacteria that were cultured for 4 h in 30 ml complete vesicle-depleted medium (prepared as described in the cells and media section) at a density of 1×10^8 cfu/ml. Upon culture, supernatants were depleted from bacteria by two centrifugation steps at $1200 \times g$ for 10 min and 0.22 μ m filtration. Cleared supernatants were then further processed by ultrafiltration and size-exclusion chromatography (SEC), as described below.

Cells and media

Human monocytic THP-1 cells (ATCC-TIB202) were cultured at 5% CO₂ and 37°C in RPMI1640 (Sigma) containing 10% fetal calf serum (FCS) (Lonza, Verviers, Belgium) with glucose (22.5%), sodium pyruvate (100 mM) and β -mercaptoethanol (25 mM). Monocytes, seeded at 0.5×10^6 cells per well in 24-wells plates, 1×10^4 per well in 96-wells plates and 1×10^7 cells in T75-flasks, were differentiated using 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72 h. Hereafter, the medium was replaced with vesicle-depleted culture medium containing 5% FCS containing glucose (22.5%), and sodium pyruvate (100 mM), hereafter referred to complete vesicle-depleted medium. Medium with 30% FCS was depleted from abundant and possibly confounding bovine MVs by combining FCS-free culture medium with vesicle-depleted medium with 30% FCS. Vesicle-depleted medium with 30% FCS was obtained by the overnight centrifugation at $100\,000 \times g$ using a 70Ti-rotor, κ -factor 44 in an Optima L-90K ultracentrifuge (both Beckman Coulter, Fullerton, CA, USA).

Infection of macrophages for MV analysis

For macrophage infections in 24-wells plates, cells were washed three times with PBS whereafter the medium was replaced with complete vesicle-depleted culture medium. Hereupon, macrophages were infected with either one of the bacteria at a multiplicity of infection of 10. After 6 h of infection, the medium was harvested, processed by two centrifugation steps at $1200 \times g$ for 10 min and 0.22 μ m filtration, and used for bead-based flow cytometry.

MV concentration and purification from conditioned medium using ultrafiltration and SEC

MVs were concentrated from bacteria-cleared supernatants by using ultrafiltration (Lobb et al. 2015) and separated from protein by SEC (Böing et al. 2014). This method for vesicle

isolation is considered one of the most appropriate methods for the isolation of mammalian extracellular vesicles as it provides with an efficient standardized method for the generation of pure intact MV populations (Böing et al. 2014; Lobb et al. 2015; Nordin et al. 2015). For ultrafiltration, the cleared supernatants were concentrated 60 times to 500 μ l in two centrifugation runs at $4000 \times g$ for 15 min at room temperature using Amicon ultra-15 10-kDa centrifugal filter units (Millipore). The method for MV purification by SEC was performed as previously described by Böing et al. (2014), with minor alterations. Briefly, a 15 ml-TELOS filtration column (Kinesis Scientific Experts, St. Neots, Cambridgeshire, UK) was stacked with 10 ml sepharose CL-2B (GE Healthcare, Uppsala, Sweden). Next, the concentrates were loaded onto the column and eluted using PBS. Twenty-four fractions of 500 μ l were collected and the MV-containing fraction (based on five pooled fractions: 7–11, previously identified to be highly enriched for MVs and negative for free proteins) was stored at -80°C until further use.

MV analysis by tunable resistive pulse sensing

The MV concentration was determined by tunable resistive pulse sensing using the qNano Gold, the IZON Control Suite Software v3.2 and the reagent kit (type RK1) for EV analysis from IZON (IZON Science Ltd, Oxford, UK). Measurements were conducted using a NP150 pore with a fixed stretch of 47 nm, a transmembrane voltage of 0.48 V (led to a baseline current of ± 100 nA) and a pressure of 6 mbar. Solution G was added (10%) to supernatants that were diluted in solution Q (1:1) and each sample was measured for 10 min and measurements were repeated when system instabilities occurred. The samples were calibrated using 114 nm polystyrene calibration beads (CPC100, IZON Science Ltd.) at a concentration of 1×10^9 particles/ml diluted in culture medium.

Flow cytometric analysis of MVs using antibody-coated latex beads

Bead-based flow cytometry was used for the semiquantitative analysis of MVs released by macrophages and bacteria. This method was previously described (Ostrowski et al. 2010; Kapustin et al. 2015) and was adopted for bacterial vesicle quantification (Volgers et al. 2017). Briefly, 4- μ m-sized aldehyde-sulfate beads were washed in MES buffer and coated with anti-CD63 for host-cell vesicles or with an antibody against one of the bacterial MVs. Antibody-coated beads were incubated overnight with 200 μ l of cleared supernatant under constant agitation at 1000 rpm at room temperature. Next, the beads were washed twice with 0.22 μ m filtered PBS with 2% (w/v) bovine serum albumin and incubated with a PE-conjugated detection antibody, for 90 min at room temperature under continuous agitation. Antibacterial antibodies were PE conjugated using a PE conjugation kit (Abcam) following the manufacturer's instructions and for the host-cell MVs, the detection was based on anti-CD81-PE labeled detection antibodies. After incubation with the PE-labeled detection antibodies, the beads were washed and suspended in 300 μ l PBS for flow cytometric analysis on a FACSCanto (BD Bioscience, Franklin Lakes, NJ, USA). The lower threshold for detection was set at 2% based on the percentage of PE-positive control beads (incubated with unconditioned culture medium). Analyses were performed using FACSDiva Software. The relative amount of MVs was calculated by the multiplication of the percentage of positive beads with the median fluorescence intensity and expressed relative to the control (as % of control).

Macrophage stimulation with MVs

Stimulation of macrophages with MVs was performed using macrophages seeded in 96-well plates. Prior to stimulation, macrophages in 96-well plates were washed three times with PBS after the medium was replaced with vesicle-depleted culture medium with 5% FCS. Then, cells were exposed to 20 μ l of the MV-containing SEC fraction for 16 h, without or in the presence of NAC (5 μ M, 5 mM or 25 mM). Hereafter, the culture supernatants were harvested and used for cytokine measurements.

Assessment of the bacterial growth and adhesion and internalization

To assess for bacterial growth during culture, the optical density at 600 nm was determined at a path length of 1 cm using a spectrophotometer (Novaspec II: Pharmacia Biotech, Uppsala, Sweden) (Sutton 2011). PMA-stimulated monocytes seeded in 24-well plates were washed three times with PBS after which vesicle-depleted culture medium was added. Next, the cells were infected with either one of the bacteria at an MOI of 10 for 4 h. After infection, the media were collected to assess bacterial survival (and for flow cytometric analysis of bacterial and host-cell MV release). To assess bacterial survival, bacteria were pelleted by centrifugation at $1200 \times g$, resuspended in 1 ml PBS, whereupon bacterial dilutions were made and plated. In addition to bacterial survival, bacterial adhesion and internalization were determined. After the supernatant was harvested, cells were washed three times with PBS and lysed by adding 0.025% saponin in distilled water for 10 min. After neutralization, by the addition of culture medium, bacterial adhesion and internalization were determined by bacterial plating. The number of adhered and internalized bacteria was then determined by counting.

Cytokine measurements

The human Ready-Set-Go TNF- α enzyme-linked immunosorbent assay kit (Affymetrix eBioscience, Santa Clara, CA, USA) was used to determine the TNF- α release according to the manufacturer's instructions.

Statistical analyses

Statistical analysis was performed using Graph-Pad Prism 5 Software (Graph-Pad, San Diego, CA, USA). Statistical dispersion was determined by calculation of the standard error of the mean. A Mann-Whitney t-test was performed for the statistical analysis of the variance between the means of two groups. P-values were considered significant when <0.05 .

RESULTS

The effect of NAC on bacterial growth

First, it was determined whether NAC affects the growth of the common respiratory pathogens NTHi, Mrc, Spn and Psa. We initially determined if the growth was affected by NAC concentrations of 5–50 mM, a concentration based on the estimated concentration of ~ 30 mM that may be achieved in the lower airway upon administration of 300 mg NAC by nebulization and which may therefore be physiologically relevant (Szkudlarek et al. 2004). As shown in Fig. 1, in the presence of 50 mM

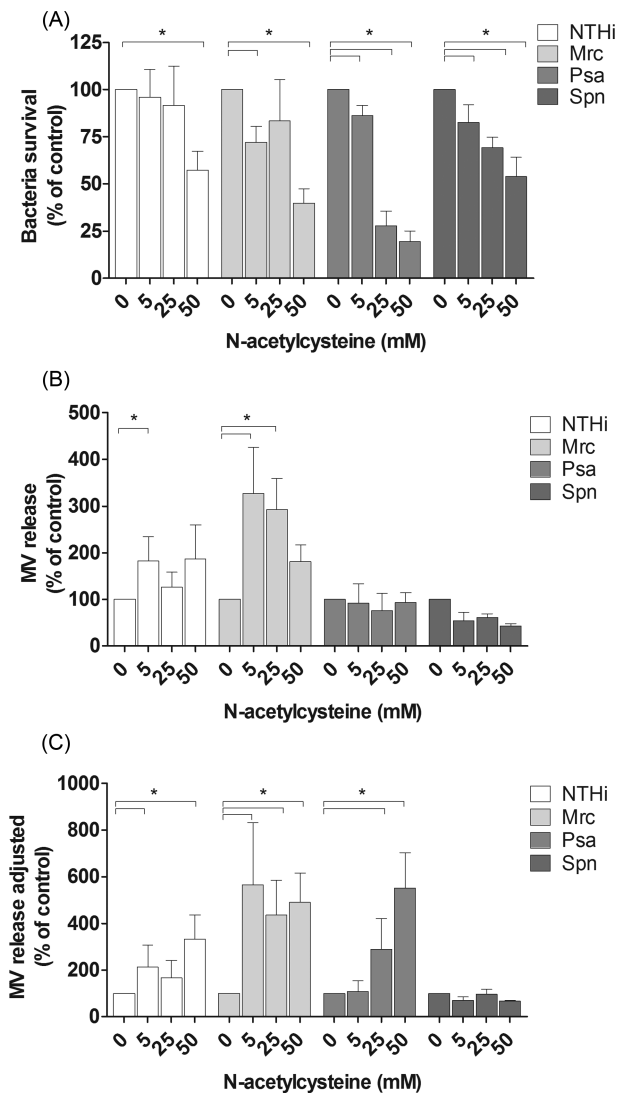


Figure 1. Bacterial growth and MV release by respiratory pathogens during bacterial culture with or without NAC. NTHi, Mrc, Spn and Psa were cultured for 6 h without or in the presence of NAC (5, 25 or 50 mM). Bacterial growth was determined with the optical density of the bacterial suspensions at 600 nm (A). Additionally, the MV release was determined by tunable resistive pulse sensing (B) and the MV release was adjusted for the number of bacteria (C). All values are presented here as percentage of the untreated control condition. ($n = 3$, $*P < 0.05$).

NAC bacterial growth was reduced by 43%, 66%, and 80% of the Gram-negative bacteria NTHi, Mrc, and Psa, respectively. Finally, the bacterial growth of the Gram-positive Spn was also reduced by 47%.

Bacterial MV release in response to NAC

Next, the effect of NAC on the release of bacterial MVs was assessed by tunable resistive pulse sensing-based analysis. We observed that the addition of NAC at a concentration of 5 mM resulted in a 2-fold and 3-fold increase in the release of NTHi and Mrc MVs, respectively (Fig. 1B). This MV release was also increased after treatment with higher concentrations, albeit to a lesser extent. Yet, since NAC markedly inhibited bacterial growth (Fig. 1A), we adjusted the release of MVs for the number of bacteria present in each culture condition. After

adjustment, the bacterial MV release was found to be increased 3-fold for NTHi and even 5-fold for Mrc (Fig. 1B). Although the bacterial MV release by Psa initially did not seem to be affected by NAC (Fig. 1A), when the percentage was adjusted for the number of bacteria the MV release was found to be increased 5-fold. Overall this suggests that although NAC significantly inhibited bacterial growth of these Gram-negative bacteria, the release of MVs per bacterium seemed to be increased in the presence of NAC. The release of MVs by Spn tended to decrease upon treatment and disappeared when adjustments for the total bacterial numbers were made suggesting that this decrease was the consequence of the reduced number of bacteria (Fig. 1A).

The effects of NAC on bacterial growth during infection

As bacterial infections of the airways are associated with prominent activation of alveolar macrophages, we examined the effects of NAC treatment on bacterial survival and MV release in an *in vitro* infection model (Martin and Frevert 2005). First, we determined bacterial growth and adhesion/internalization after macrophage infection with either one of the bacteria, and determined how this was affected by 25 mM NAC. NAC treatment almost completely prevented bacterial growth and adhesion/internalization of NTHi and Mrc (Fig. 2A and B). Treatment of Psa-infected macrophages had inconsistent effects on the bacterial growth and hardly affected adhesion/internalization (Fig. 2A and B). Finally, treatment of Spn led to a ~50% reduction of the bacterial growth but did not affect the adherence/internalization (Fig. 2A and B).

The effect of NAC on the release of bacterial and host cell-derived MVs during macrophage infection

Next, we studied if bacterial MV shedding during macrophage infection was affected by NAC. In the first set of experiments, tunable resistive pulse sensing was used to quantify MVs in a bacterial monoculture. However, during infection the MV population released consisted of both bacterial and host-cell MVs. Therefore, a bead-based flow-cytometry assay for the semiquantitative determination of MVs was applied here. Unfortunately, we failed to establish this platform for Spn so we could not quantify the vesicle release for this species in this setting.

At a concentration of 25 mM, NAC increased the MV release by NTHi and Mrc (Fig. 3A). When adjustments were made for the total number of bacteria, this increase was 5-fold for NTHi and 10-fold for Mrc ($p \leq 0.05$ vs control, Fig. 3B). Regarding Psa, when adjustments were made, the MV release was found to increase on treatment with 25 mM NAC (Fig. 3B).

MV shedding during infection is a dynamic process that occurs by both bacteria and the host. As it has been shown previously that host cell-derived MVs also have immunostimulatory properties (Qazi *et al.* 2010; Kulshreshtha *et al.* 2013; Soni *et al.* 2016), we additionally determined the effect of NAC on the release of CD63⁺/CD81⁺ MVs by macrophages. Treatment with 25 mM NAC significantly reduced the release of host-derived vesicles by macrophages, irrespective of the bacteria they were infected with (Fig. 4).

The proinflammatory response to MVs is reduced by NAC

In earlier experiments, we demonstrated that bacterial MVs induce the release of proinflammatory cytokines by naïve

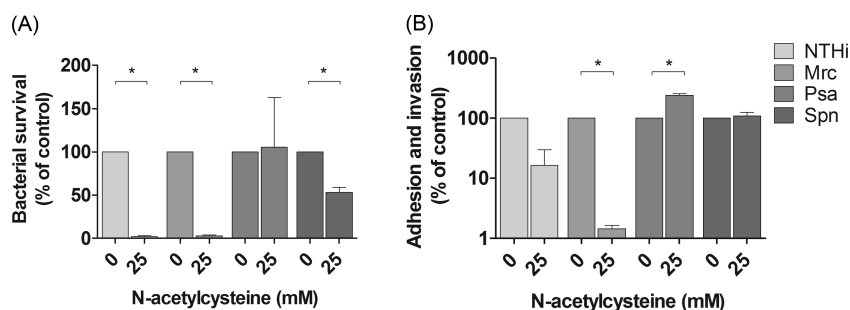


Figure 2. The effect of NAC on bacterial survival and adhesion and internalization of respiratory pathogens during macrophage infection. Bacterial survival (A) and adhesion/internalization (B) were determined after 4 h of infection with NTHi, Mrc, Spn and Psa without or with 25 mM NAC. Bacteria were quantified by plating and the counts are expressed as percentage of the untreated control ($n = 3$, $*P < 0.05$).

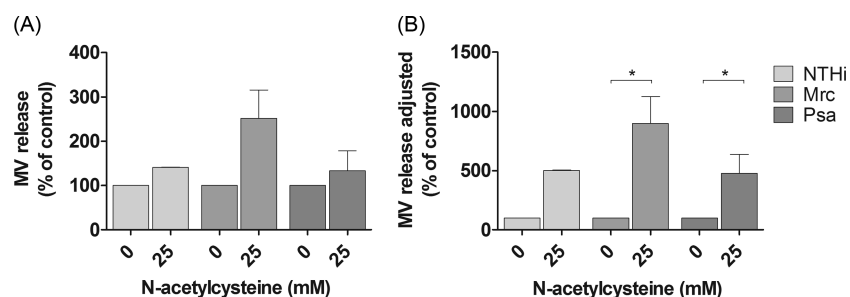


Figure 3. Bacterial MV release by respiratory bacteria during infection without or with 25 mM NAC. The bacterial MV release after 4 h of infection in the presence 25 mM NAC or under control conditions was determined by bead-based flow cytometry. The relative counts are presented here as a percentage of the untreated control (A). Moreover, the release was adjusted for the total number of bacteria present (B) ($n = 3$, $*P < 0.05$).

macrophages. Here, we determined if this proinflammatory response induced by MVs from NTHi, Mrc, Spn and Psa was affected by NAC treatment. As shown in Fig. 5A, all MV populations except those of Spn induced the release of TNF- α by naive macrophages, albeit with different potencies. The response of macrophages to MVs was dose dependently reduced in the presence of NAC for all conditions except for Spn. Importantly, we determined that this decreased release of TNF- α was not the result of a treatment-induced cytotoxicity as NAC treatment was not found to have cytotoxic effects (Fig. 5B). Interestingly, MVs from NTHi and Mrc showed to have cytotoxic effects that could be prevented by NAC treatment.

DISCUSSION

NAC is best known for its mucolytic properties, and because of its additional antioxidant and anti-inflammatory properties, it is an attractive drug in COPD therapy (van Overveld et al. 2005; Poole 2006; Sadowska et al. 2006; Cazzola et al. 2015; Poole, Chong and Cates 2015). Moreover, bacteriostatic characteristics have been assigned to NAC in several studies (Riise et al. 2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004; Hafez et al. 2009; Zhao and Liu 2010). In this study, we confirmed this bacteriostatic effect as the growth of several respiratory pathogens was markedly inhibited by NAC. Therefore, as many exacerbations result from bacterial infections, the therapeutic effect of NAC may be beyond its mucolytic and anti-inflammatory and anti-oxidative properties. Additionally, in this study we demonstrate that NAC, despite an enhancement of the release of proinflammatory MVs by the Gram-negative bacteria used in this study, suppressed the release of inflammatory cytokines by naive macrophages in response to these MVs.

There is a great demand for new treatments that relieve airway obstruction and inflammatory processes in patients with COPD (Barnes 2003). Treatment with mucolytics, most prominently NAC, has been shown to alleviate these symptoms (Cazzola et al. 2015; Poole, Chong and Cates 2015). Moreover, (part of) these patients frequently suffer from severe exacerbations which are mostly caused by respiratory infections. Recently, Cazzola et al. (2015) concluded from their meta-analysis that patients with COPD or bronchitis who received oral NAC treatment had a decreased risk of exacerbations, although higher doses were required in patients with airway obstruction. There are indications that this protective effect of NAC may not only rely on its mucolytic, anti-oxidant or anti-inflammatory effects, but also on its antibacterial activity. One study by Riise et al. (1994) shows that COPD patients who received oral NAC therapy had a significant reduction of the intrabronchial bacterial numbers. This antibacterial activity has already been demonstrated in the 1970s by Parry and Neu (1977), who demonstrated that NAC inhibited the growth of both Gram-negative and Gram-positive bacteria. Likewise, we also found that NAC caused a dose-dependent growth reduction of all bacteria tested. Also, during infection of naive macrophages, survival of NTHi and Mrc was significantly reduced in the presence of NAC. Adhesion of these two Gram-negative bacteria was markedly diminished by NAC. This is in accordance by findings by others, who also demonstrated that the presence of NAC significantly reduced the ability of Spn, NTHi or Psa to adhere to epithelial cells, *in vitro* (Niederman et al. 1983; Zheng et al. 1999; Riise et al. 2000; Olofsson, Hermansson and Elwing 2003; Hafez et al. 2009). These data suggest a possible role for NAC beyond its well-known effects.

The release of MVs, which have been shown to be important vehicles for the simultaneous delivery of many effector molecules to host cells, is a common feature for both

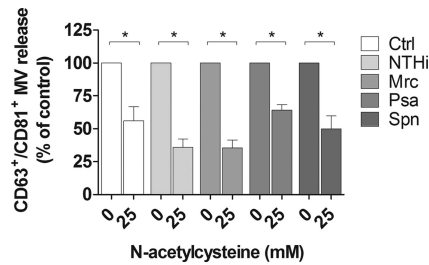


Figure 4. MV release by macrophages after infection with either one of the respiratory pathogens in the presence or absence of 25 mM NAC. The release of CD63⁺/CD81⁺ MVs by macrophages after 4 h of infection with either one of the bacteria (multiplicity of infection of 10) in the presence or absence of 25 mM NAC was determined by bead-based flow cytometry. The relative counts are expressed here as a percentage of the untreated control ($n = 3$, $*P < 0.05$).

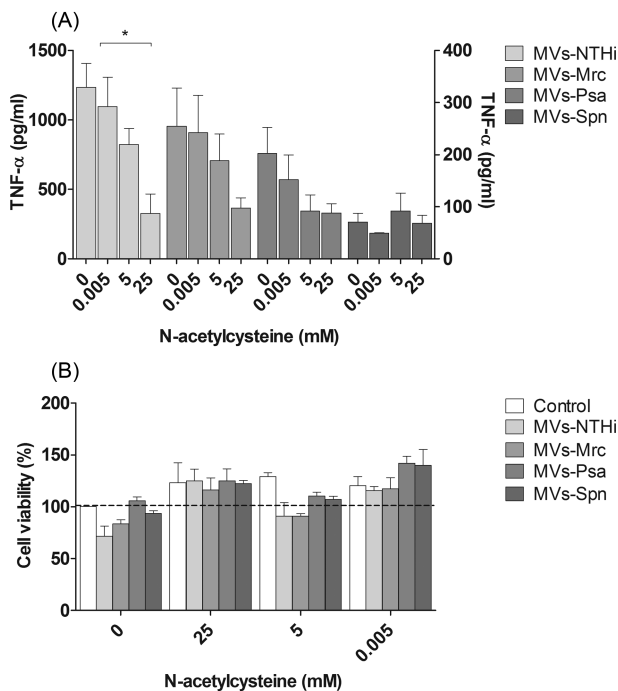


Figure 5. The effect of NAC on the TNF- α response by naive macrophages after stimulation with bacterial MVs. Macrophages were exposed to bacterial MVs without or in the continuous presence of NAC (0.005, 5 or 25 mM) overnight, where after the release of TNF- α was determined (data for Mrc-, Psa-, and Spn-MVs are plotted on the right y-axis) (A). The cell viability after overnight MV exposure and/or NAC treatment was determined by a MTT assay (B). The results are presented as the mean \pm SEM ($n = 3$, $*P < 0.05$).

Gram-negative and Gram-positive bacteria (Brown et al. 2015; Schwechheimer and Kuehn 2015). MVs may influence the course of infection and the host immune response by presenting pathogen-associated molecular patterns and antigens to their respective host receptors. A potential involvement of bacterial MVs in the pathogenesis of lung disease is indicated by several studies that demonstrate that bacterial MVs are present in infected tissues (Bauman and Kuehn 2006; Perez Vidakovics et al. 2010; Ren et al. 2012). Moreover, it has been demonstrated that bacterial MVs are well able to activate immune cells and induce the release of proinflammatory cytokines. Additionally, bacterial MVs have been shown to facilitate replication of *Legionella pneumophila* in infected macrophages or to induce emphysema via IL-17A-mediated neutrophilic inflammation (Kim et al. 2015; Jung et al. 2016). Thus, inhibition of bacterial MV release might be

beneficial for the course of pulmonary disease associated with infection (and other infection-related diseases). Here we examined the effects of NAC on the release of MVs by common respiratory pathogens. We provide evidence that treatment markedly enhanced the release of these vesicles by all Gram-negative bacteria when corrected for the number of bacteria. In contrast, no effects on the MV release by the Gram-positive Spn were observed.

The proposed mechanism by which NAC inhibits bacterial growth is by inhibiting the cysteine utilization by bacteria (Parry and Neu 1977). Additionally, this may also explain how NAC triggers the release of MVs as a recent study showed that the inhibition of cysteine utilization in *Neisseria meningitidis* cultures had bacteriostatic effects and promoted the release of bacterial MVs (van de Waterbeemd et al. 2013). That the bacteriostatic effects of NAC are likely due to the inhibition of cysteine utilization might also explain the species-specific effects observed. Since the cysteine requirements differ among bacteria and can even vary between strains, these differences may affect the bacterial sensitivity to NAC (Guédon and Martin-Verstraete 2006).

We also showed that particularly MVs from the Gram-negative bacteria stimulated the release of TNF- α by naive macrophages. This is in accordance with earlier studies demonstrating a MV-induced release of proinflammatory cytokines and chemokines *in vitro* as well as *in vivo* (Bauman and Kuehn 2006; Schaar et al. 2011; Sharpe, Kuehn and Mason 2011; Lee et al. 2012; Park et al. 2013). Thus, in view of the enhanced release of MVs by our Gram-negative bacteria, the therapeutic potential of NAC may be limited as it may hereby enhance the pulmonary inflammation. However, when we stimulated naive macrophages with bacterial MVs, the release of TNF- α could be dose-dependently inhibited by increasing concentrations of NAC. This suggests that NAC might be able to counteract its proinflammatory effect due to the enhanced bacterial MV release by inhibiting the subsequent release of proinflammatory cytokines by activated immune cells. Additional studies are warranted to further examine the net effect of NAC in pulmonary bacterial infections.

Although several *in vitro* studies have shown that NAC has potent anti-oxidant and anti-inflammatory (as reviewed by Sadowska, Manuel-Y-Keenoy and De Backer 2007), as well as bacteriostatic properties (Parry and Neu 1977; Riise et al. 2000; Olofsson, Hermansson and Elwing 2003; Huynh et al. 2004; Hafez et al. 2009; Zhao and Liu 2010), this was mostly at concentrations which are hard to achieve by oral administration. For example, upon oral administration of 600 mg thrice daily for 5 days, NAC could not be detected in the epithelial lining fluid, bronchoalveolar lavage fluid or lung tissue (Cotgreave et al. 1987; Bridgeman et al. 1994). Yet, for certain respiratory conditions, such as idiopathic pulmonary fibrosis and cystic fibrosis, NAC is administered by nebulization (350 mg, twice daily), whereby a high local bioavailability is reached (Homma et al. 2012; Tam et al. 2013). In view of the present data, which suggest that COPD patients may also benefit from high local NAC concentrations, nebulization might form an attractive alternative in the treatment of these patients.

To summarize, in the current study we demonstrated that high NAC concentrations exert bacteriostatic effects on NTHi, Mrc, Spn and Psa cultures and on NTHi and Mrc during macrophage infection. On the other hand, this inhibition of the bacterial growth was accompanied by an increased MV release. However, as our results also show that NAC markedly reduces the proinflammatory response to MVs, it is likely that the increased MV release is of little consequence. This may implicate that NAC administration by nebulization may have a positive

treatment outcome during airway infections, such as bacterial exacerbations of COPD, but only when administered at a high concentration. Future research will be required to study these effects *in vivo* and to determine the long-term effects of NAC treatment on respiratory infections and exacerbations.

ACKNOWLEDGEMENTS

The authors wish to thank Dr J. P. Hays from the Erasmus University Medical Centre (Erasmus MC), Rotterdam, the Netherlands for kindly providing the rabbit antisera to *Moraxella catarrhalis* used in this publication.

FUNDING

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Conflict of interest. None declared.

REFERENCES

- Barnes PJ. Chronic obstructive pulmonary disease: new treatments for COPD. *Thorax* 2003;**58**:803–8.
- Bauman SJ, Kuehn MJ. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes Infect* 2006;**8**:2400–8.
- Böing AN, van der Pol E, Grootemaat AE et al. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014;**3**:23430.
- Bridgeman MM, Marsden M, Selby C et al. Effect of N-acetyl cysteine on the concentrations of thiols in plasma, bronchoalveolar lavage fluid, and lung tissue. *Thorax* 1994;**49**:670–5.
- Brown L, Wolf JM, Prados-Rosales R et al. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol* 2015;**13**:620–30.
- Cazzola M, Calzetta L, Page C et al. Influence of N-acetylcysteine on chronic bronchitis or COPD exacerbations: a meta-analysis. *Eur Respir Rev* 2015;**24**:451–61.
- Cotgreave IA, Eklund A, Larsson K et al. No penetration of orally administered N-acetylcysteine into bronchoalveolar lavage fluid. *Eur J Respir Dis* 1987;**70**:73–7.
- Desai H, Eschberger K, Wrona C et al. Bacterial colonization increases daily symptoms in patients with chronic obstructive pulmonary disease. *Ann Am Thorac Soc* 2014;**11**:303–9.
- Guédon E, Martin-Verstraete I. Cysteine metabolism and its regulation in bacteria. *Microbiol Monogr* 2006;**5**:195–218.
- Hafez MM, Aboulwafa MM, Yassien MA et al. Activity of some mucolytics against bacterial adherence to mammalian cells. *Appl Biochem Biotechnol* 2009;**158**:97–112.
- Hauber HP, Foley SC, Hamid Q. Mucin overproduction in chronic inflammatory lung disease. *Can Respir J* 2006;**13**:327–35.
- Homma S, Azuma A, Taniguchi H et al. Efficacy of inhaled N-acetylcysteine monotherapy in patients with early stage idiopathic pulmonary fibrosis. *Respirology* 2012;**17**:467–77.
- Huynh HQ, Couper RTL, Tran CD et al. N-acetylcysteine, a novel treatment for *Helicobacter pylori* infection. *Dig Dis Sci* 2004;**49**:1853–61.
- Jung AL, Stoiber C, Herkt CE et al. Legionella pneumophila-derived outer membrane vesicles promote bacterial replication in macrophages. *PLoS Pathog* 2016;**12**:e1005592.
- Kapustin AN, Chatrou MLL, Drozdov I et al. Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. *Circ Res* 2015;**116**:1312–23.
- Kim Y-S, Lee W-H, Choi E-J et al. Extracellular vesicles derived from Gram-negative bacteria, such as *Escherichia coli*, induce emphysema mainly via IL-17A-mediated neutrophilic inflammation. *J Immunol* 2015;**194**:3361–8.
- King PT, MacDonald M, Bardin PG. Bacteria in COPD; their potential role and treatment. *Transl Respir Med* 2013;**1**:13.
- Kulshreshtha A, Ahmad T, Agrawal A et al. Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol* 2013;**131**:1194–203.
- Lee JC, Lee EJ, Lee JH et al. *Klebsiella pneumoniae* secretes outer membrane vesicles that induce the innate immune response. *FEMS Microbiol Lett* 2012;**331**:17–24.
- Lobb RJ, Becker M, Wen SW et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles* 2015;**1**:1–11.
- Lopez AD, Mathers CD, Ezzati M et al. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 2006;**367**:1747–57.
- Marin A, Garcia-Aymerich J, Sauleda J et al. Effect of bronchial colonisation on airway and systemic inflammation in stable COPD. *COPD* 2012;**9**:121–30.
- Martin TR, Frevert CW. Innate immunity in the lungs. *Proc Am Thorac Soc* 2005;**2**:403–11.
- Miravittles M. Cough and sputum production as risk factors for poor outcomes in patients with COPD. *Respir Med* 2011;**105**:1118–28.
- Montes De Oca M, Halbert RJ, Lopez MV et al. The chronic bronchitis phenotype in subjects with and without COPD: The PLATINO study. *Eur Respir J* 2012;**40**:28–36.
- Niedermaier MS, Rafferty TD, Sasaki CT et al. Comparison of bacterial adherence to ciliated and squamous epithelial cells obtained from the human respiratory tract. *Am Rev Respir Dis* 1983;**127**:85–90.
- Nordin JZ, Lee Y, Vader P et al. Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. *Nanomed-Nanotechnol* 2015;**11**:879–83.
- Olofsson A-C, Hermansson M, Elwing H. N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. *Appl Environ Microb* 2003;**69**:4814–22.
- Ostrowski M, Carmo NB, Krumeich S et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 2010;**12**:13–9.
- Park KS, Lee J, Jang SC et al. Pulmonary inflammation induced by bacteria-free outer membrane vesicles from *Pseudomonas aeruginosa*. *Am J Resp Cell Mol* 2013;**49**:637–45.
- Parry MF, Neu HC. Effect of N-acetylcysteine on antibiotic activity and bacterial growth *in vitro*. *J Clin Microbiol* 1977;**5**:58–61.
- Pauwels R. Global initiative for chronic obstructive lung diseases (GOLD): time to act. *Eur Respir J* 2001;**18**:901–2.
- Perez Vidakovics MLA, Jendholm J, Mörgelin M et al. B cell activation by outer membrane vesicles—a novel virulence mechanism. *PLoS Pathog* 2010;**6**:e1000724.
- Poole P, Chong J, Cates CJ. Mucolytic agents versus placebo for chronic bronchitis or chronic obstructive pulmonary disease. *Cochrane Db Syst Rev* 2015;**7**:CD001287.
- Poole PJ. Role of mucolytics in the management of COPD. *Int J Chron Obstruct Pulmon Dis* 2006;**1**:123–8.

- Qazi KR, Torregrosa Paredes P, Dahlberg B et al. Proinflammatory exosomes in bronchoalveolar lavage fluid of patients with sarcoidosis. *Thorax* 2010;**11**:1016–24.
- Ren D, Nelson KL, Uchakin PN et al. Characterization of extended co-culture of non-typeable *Haemophilus influenzae* with primary human respiratory tissues. *Exp Biol Med* 2012;**237**:540–7.
- Riise GC, Larsson S, Larsson P et al. The intrabronchial microbial flora in chronic bronchitis patients: A target for N-acetylcysteine therapy? *Eur Respir J* 1994;**7**:94–101.
- Riise GC, Qvarfordt I, Larsson S et al. Inhibitory effect of N-acetylcysteine on adherence of *Streptococcus pneumoniae* and *Haemophilus influenzae* to human oropharyngeal epithelial cells in vitro. *Respiration* 2000;**67**:552–8.
- Sadowska AM, Manuel-Y-Keenoy B, De Backer WA. Antioxidant and anti-inflammatory efficacy of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review. *Pulm Pharmacol Ther* 2007;**20**:9–22.
- Sadowska AM, Verbraecken J, Darquennes K et al. Role of N-acetylcysteine in the management of COPD. *Int J Chron Obstruct Pulmon Dis* 2006;**1**:425–34.
- Schaar V, De Vries SPW, Perez Vidakovics MLA et al. Multicomponent *Moraxella catarrhalis* outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells. *Cell Microbiol* 2011;**13**:432–49.
- Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* 2015;**13**:605–19.
- Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N Engl J Med* 2008;**359**:2355–65.
- Sharpe SW, Kuehn MJ, Mason KM. Elicitation of epithelial cell-derived immune effectors by outer membrane vesicles of nontypeable *haemophilus influenzae*. *Infect Immun* 2011;**79**:4361–9.
- Sheffner AL. The reduction in vitro in viscosity of mucoprotein solutions by a new mucolytic agent, N-acetyl-L-cysteine. *Ann N Y Acad Sci* 1963;**106**:298–310.
- Soni S, Wilson MR, O'Dea KP et al. Alveolar macrophage-derived microvesicles mediate acute lung injury. *Thorax* 2016;**71**:1020–9.
- Sutton S. Measurement of microbial cells by optical density. *J Valid Techn* 2011;**17**:46–9.
- Szkudlarek U, Zdziechowski A, Witkowski K et al. Effect of inhaled N-acetylcysteine on hydrogen peroxide exhalation in healthy subjects. *Pulm Pharmacol Ther* 2004;**17**:155–62.
- Tam J, Nash EF, Ratjen F et al. Nebulized and oral thiol derivatives for pulmonary disease in cystic fibrosis. *Cochrane Db Syst Rev* 2013;**7**:CD007168.
- van de Waterbeemd B, Zomer G, van den IJssel J et al. Cysteine depletion causes oxidative stress and triggers outer membrane vesicle release by *Neisseria meningitidis*; implications for vaccine development. *PLoS One* 2013;**8**:e54314.
- van Overveld FJ, Demkow U, Górecka D et al. New developments in the treatment of COPD: comparing the effects of inhaled corticosteroids and N-acetylcysteine. *J Physiol Pharmacol* 2005;**56** Suppl 4:135–42.
- Volgers C, Benedikter BJ, Grauls E et al. Bead-based flow-cytometry for semi-quantitative analysis of complex membrane vesicle populations released by bacteria and host-cells. *Microbiol Res* 2017;**200**:25–32.
- Zhao T, Liu Y. N-acetylcysteine inhibit biofilms produced by *Pseudomonas aeruginosa*. *BMC Microbiol* 2010;**10**:140.
- Zheng CH, Ahmed K, Rikitomi N et al. The effects of S-carboxymethylcysteine and N-acetylcysteine on the adherence of *Moraxella catarrhalis* to human pharyngeal epithelial cells. *Microbiol Immunol* 1999;**43**:107–13.