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The host immune response contributes to *Haemophilus influenzae* virulence☆

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Summary
Background: There is compelling evidence that infections with non-typeable *Haemophilus influenzae* (NTHi) are associated with exacerbations in COPD patients. However, NTHi has also been isolated frequently during clinically stable disease. In this study we tested the hypothesis that genetically distinct NTHi isolates obtained from COPD patients differ in virulence which could account for dissimilarities in the final outcome of an infection (stable vs. exacerbation).
Results: NTHi isolates ($n = 32$) were obtained from stable COPD patients, or during exacerbations. Genetically divergent NTHi isolates were selected and induction of inflammation was assessed as an indicator of virulence using different *in vitro* models. Despite marked genomic differences among NTHi isolates, *in vitro* studies could not distinguish between NTHi isolates based on their inflammatory capacities. Alternatively, when using a whole blood assay results demonstrated marked inter-, but not intra-individual differences in

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cytokine release between healthy volunteers irrespective of the origin of the NTHi isolate used.
Conclusion: Results suggest that the individual immune reactivity might be an important predictor for the clinical outcome (exacerbation vs. no exacerbation) following NTHi infection.

Introduction

Acute exacerbations (AE) are responsible for an enormous burden in the clinical course of chronic obstructive pulmonary disease (COPD) [1,2]. Regarding the cause of these AEs, a strong link between AEs and bacterial as well as viral infections has been shown [2—5]. In particular the non-typeable *Haemophilus influenzae* (NTHi) Gram-negative bacterium has been associated frequently with acute exacerbations [2,6,7]. However, a direct cause-effect relationship is lacking since NTHi has been isolated from COPD patients not only during AEs but also in clinically stable periods [8]. This raises the important question why some patients develop an exacerbation following a respiratory tract infection while others remain stable. To answer this, previous research has primarily focused on differences in bacterial load, the location of the infection or the acquisition of a new bacterial strain [9—11]. Moreover, it has been speculated that differences in virulence account for dissimilarities in disease pathogenesis [12]. Genomic analysis of NTHi identified candidate genes possible important for bacterial pathogenesis. For example, genetic islands were more prevalent in pathogenic compared to non-pathogenic NTHi strains [13,14]. We hypothesize that among genetic distinct NTHi strains, some strains possess characteristics that makes them more pro-inflammatory than others. As NTHi does not produce specific toxins [15], virulence of a strain can be defined as the intensity of the inflammatory reaction following infection using different *in vitro* models. In the present study we examined whether there are significant differences in virulence between clinical derived, genetically distinct NTHi isolates. The virulence of genetically unrelated isolates was compared in an *in vitro* setting by assessing the inflammatory response by a macrophage-like cell line and airway epithelial cells following exposure to the different isolates. Additionally, a clinically more relevant approach was used to assess the ability of these isolates to induce an immune response in human immune cells. We examined the potency of the isolates to induce the release of various pro-inflammatory cytokines in a whole blood assay using blood of healthy young volunteers.

Materials and methods

Bacterial isolates and growth conditions

Non-typeable *Haemophilus influenza* isolates ($n = 32$) were obtained from sputum collected from COPD patients referred for integrated assessment and pulmonary rehabilitation at CIRO+, a centre of expertise for chronic organ failure in Horn, the Netherlands. The recruitment criteria for study participation included a clinical diagnosis of COPD, a minimal age of 40 years and a clinically stable state of disease during 4 weeks. Exclusion criteria involved the diagnoses of asthma or other respiratory diseases, instable heart and/or other chronic organ failure, malignancy or use of oral glucocorticosteroids or other immune-suppressive medication. Sputum samples were obtained at inclusion into the study and during periods of AE's. An AE was diagnosed in case of increased dyspnea and changes in sputum volume or purulence. Sputum was used for routine bacterial culture. NTHi isolates were identified with standard laboratory procedures and stored in −80 °C. The ATCC 49247 strain was included in the study as laboratory reference NTHi strain. Bacterial isolates of NTHi were grown on chocolate blood agar plates supplemented with vitalex (Oxoid, Wesel, Germany) to obtain a starting inoculum of 0.5 McFarland (1.5×10^8 cfu/mL) in cell culture medium without FCS for the *in vitro* assays. For some assays, 0.5 McFarland suspensions were heat inactivated (HI) at 65 °C for 30 min.

Pulsed-field gel electrophoresis (PFGE)

Selected isolates were subjected to PFGE essentially as described previously [16]. Restriction digestion was performed with SmaI (Invitrogen), and the reference strain *Staphylococcus aureus* PS 47 used as molecular weight marker. Patterns were analyzed with Dice comparison and unweighted pair-group matching analysis with Gel Compare II 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). The position tolerance was set on 2.0 and 87.0% similarity was used as breakpoint to cluster the bands [17]. Each cluster of related strains was defined numerically in separated groups.

Determination of NTHi virulence

Adhesion in an airway epithelial cell infection model

Type II alveolar epithelial cells (A549 cell line, ATCC CCl-185) were cultured according to the supplier's recommendations, seeded at a density of 10^5 cells/mL and grown till confluence in 24-well plates (Becton Dickinson, NJ, USA). After 48 h, bacterial adherence to A549 cells was assessed essentially as previously described [18]. In short, cells were incubated for 4 h with a selection of NTHi isolates ($n = 10$) derived from COPD patients, using starting inocula of 0.5 McFarland in RPMI (Gibco, Invitrogen, Grand Island, NY, USA) without FCS (Lonza, Verviers, Belgium). Next, non-adherent bacteria were removed by washing with PBS, and the cellular content was harvested with 1% saponin in PBS (1 mL). Bacterial numbers in both the starting inoculum as well as saponin/PBS solution were quantified by spiral

plating and the amount of adherent bacteria was expressed as percentage of the starting inoculum.

Cytokine production and CXCL-8 and ICAM-1 mRNA expression

Measurements of either cytokine production or CXCL-8/ICAM-1 mRNA expression were preformed essentially as described before [19]. A549 cells and 0.5 McFarland suspensions of different NTHi isolates ($n = 10$) were prepared as described above and co-cultured for 6 h. Supernatant was collected for analysis of CXCL-8 production by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. A549 cells were collected and CXCL-8 and ICAM-1 mRNA expression was determined by RT-qPCR as described before [20]. Relative gene expressions were normalized to the expression of β-actin according to the $2^{-\Delta Ct}$ method [21].

NTHi-induced release of inflammatory cytokines by macrophages

THP-1 cells (ATCC-TIB202 a monocytic leukemic cell line), were maintained according to the supplier's recommendations. Differentiation into a macrophage phenotype was achieved by an extended protocol which drives cells towards a more differentiated macrophage phenotype that closely resemble monocyte-derived macrophages (MDM) [22]. Following this phorbol 12-myristate 13-acetate (PMA) differentiation period, cells were seeded in a 24-well plate at a density of 5×10^4 cells/well in 900 μL in Dulbecco's Modified Eagle Medium-F12 (Ham) (DMEM, Gibco Invitrogen, Grand Island, NY, USA) with 0.1% FCS. Five hours later 100 μL of heat-inactivated 0.5 McFarland NTHi solutions ($n = 18$) in DMEM without FCS were added to the cells. After 24 h cell-free supernatants were collected and stored at −80 °C until further processing. Cytokine (TNF-α, IL-6 and IL-1β) production in the supernatants was determined with commercially available ELISA kits (eBioscience, Vienna, Austria).

Inflammatory responsiveness towards NTHi strains in a whole blood assay

Next, the potency of the different strains to induce the release of different inflammatory cytokines was tested in an *ex vivo* assay. This whole blood assay (WBA) was performed as described before [23]. Whole blood was collected in heparin containing vacutainer tubes (BD Vacutainer, Plymouth, UK) from 24 healthy, young, non-smoking volunteers (11M/13F, age 21−31 y). The study was approved by the local ethics committee and informed consent was obtained from all study participants. For stimulation with NTHi, starting inocula of 0.5 McFarland in RPMI ($n = 18$) were prepared and heat-inactivated. Aliquoted blood of each volunteer was subsequently stimulated with all inactivated bacterial suspensions (100 μL). After 24 h the cell-free supernatants were collected by centrifugation and stored at −80 °C until further processing. Cytokine production in the supernatants was determined with commercially available ELISA kits for TNF-α, IL-6 en IL-1β (eBioscience, Vienna, Austria). The limits of sensitivity were 4 pg/mL for TNF-α, 2 pg/mL for IL-6 and 4 pg/mL IL-1β.

Statistics

To determine significant difference in virulence between the NTHi isolates, data were analyzed using independent *t*-test/One way ANOVA. Data are shown as mean ± SEM for the *in vitro* models. For all analyses $p < 0.05$ was regarded as statistical significant.

Results

Genetic relatedness of NTHi isolates

NTHi isolates were collected from COPD patients participating in an eight-week inpatient pulmonary rehabilitation program at CIRO+. During the rehabilitation period, the risk of cross contamination between patients is present. Therefore, we initially analysed the NTHi isolates at a genomic level by PFGE to exclude the possibility that all isolates examined were members of the same strain. The genomic fingerprints of clinical NTHi isolates obtained from different COPD patients and the ATCC strain 49247 are shown in Fig. 1. PFGE fingerprints were analysed through the construction of a dendrogram, which shows that the 32 different isolates clustered into 22 different major groups at the 87% similarity breakpoint, implicating limited cross contamination between patients and a high genetic diversity among NTHi isolates. One dendrogram cluster contains identical profiles or highly related profiles. Based on this genetic diversity we selected 18 NTHi isolates for further experiments. All selected strains were classified into distinct clusters and isolated from 18 different COPD patients (Table 1) in order to achieve the highest possible diversity between isolates.

Adherence to lung epithelial cells

As NTHi initiates infections by adhering to the airway epithelium [24], bacterial adhesion of a selection of the NTHi isolates to airway epithelial cells was determined using an *in vitro* model system. Adhesion was examined following incubation of A549 cells with the selected NTHi isolates for 4 h. Although variation in adhesion is observed between the different strains used (especially strain 2), results showed that overall there were no major differences in adhesion between distinct NTHi isolates (Fig. 2).

Inflammatory response in lung epithelial cells

Next, we used the ability of a selection of the NTHi isolates to induce the expression of the inflammatory defense genes CXCL-8 and ICAM-1 in airway epithelial cells as an indicator for the virulence of the strains. Hence, A549 cells were stimulated with the selected NTHi isolates for 6 h. RT-qPCR data revealed a low constitutive expression of both ICAM-1 and CXCL-8 mRNA in A549 cells. This expression was markedly enhanced after stimulation with NTHi isolates.

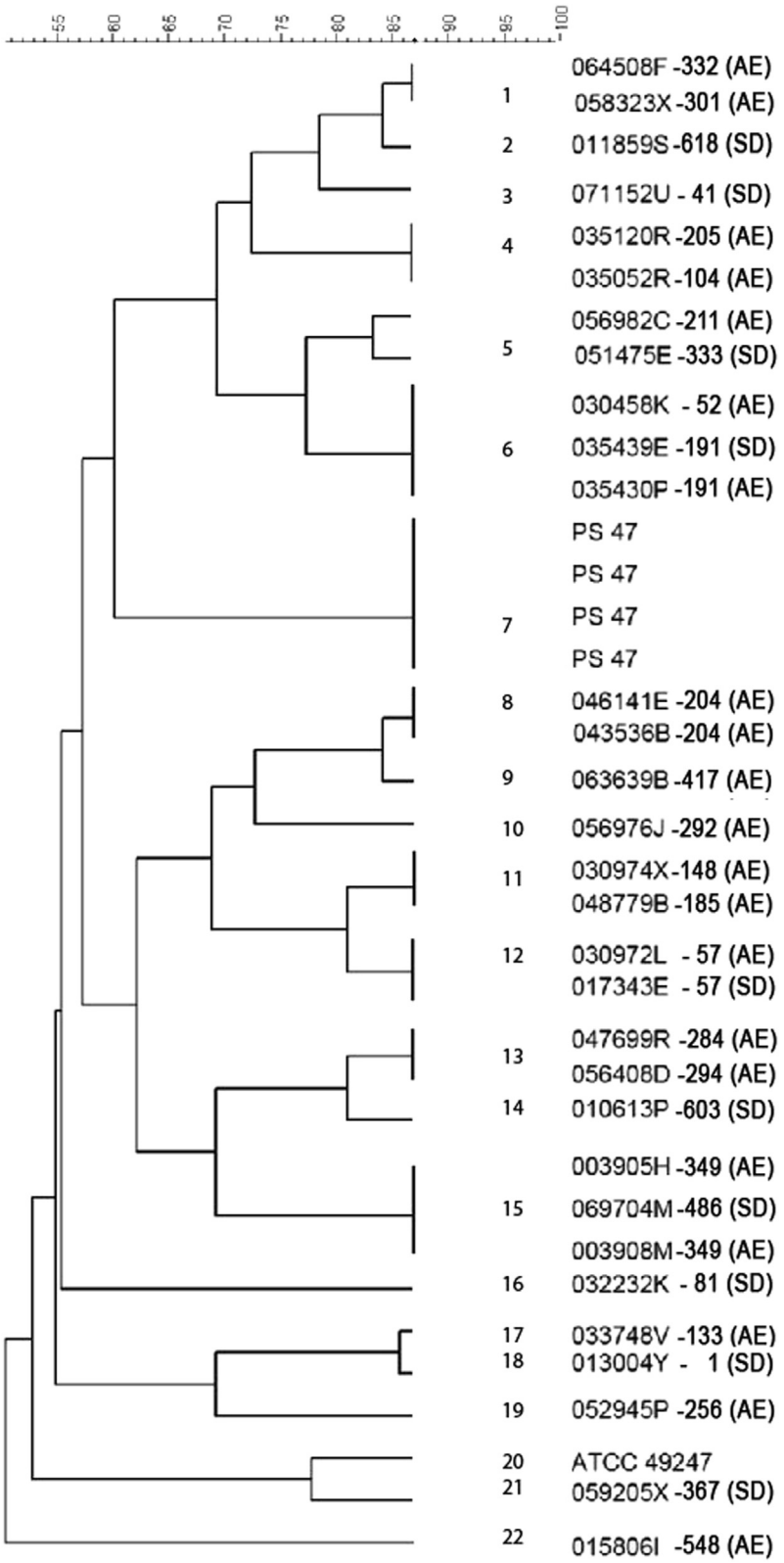

Figure 1 Dendrogram of NTHi isolates from different COPD patients. Dendrogram was constructed based on the fingerprints obtained by PFGE using Gel Compare and the unweighted-pair group method of average linkage (UMPGA). It illustrates the patterns grouping into 22 unrelated clusters based on the breakpoint of 87% similarity. Isolates and their origin are shown in the right column. 5—10 AE and 5—8 SD isolates were selected from unrelated clusters for further experiments.

Table 1 Characteristics of COPD patients with sputum samples containing selected NTHi.

Characteristics	NTHi $n = 18$ Mean $\pm$ SD
Gender	10M/8F
Age	66.27 $\pm$ 6.70
Gold stage	2.80 $\pm$ 1.08
BMI	2.43 $\pm$ 0.94
FEV1%	51.36 $\pm$ 18.40
Smoking (pack years)	10.70 $\pm$ 13.06

However, the induced expression was not significantly different between the different NTHi isolates (Fig. 3). In line with these results is our finding that the NTHi-induced CXCL-8 release was comparable when A549 cells were stimulated with the selected isolates (Fig. 3).

Inflammatory response in human macrophages

As macrophages are the sentinel cells for the initiation of the innate immune response, the capacity of NTHi to elicit the release of various cytokines by macrophages was assessed [25]. The ability to produce the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 of PMA-differentiated THP-1 was compared as an indicator of virulence. Yet, no significant differences were observed in the amounts of inflammatory cytokines released by differentiated THP-1 cells following stimulation with either of the NTHi strains (Fig. 4).

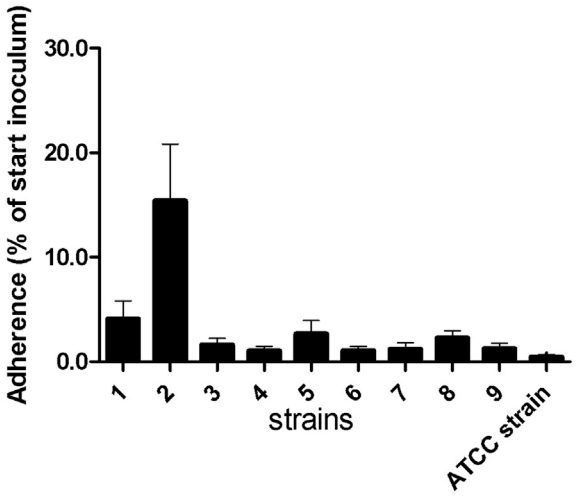

Figure 2 Bacterial adherence of NTHi isolates to human airway epithelial cells (A549). Adherence was determined after infecting a confluent layer of A549 cells for 4 h with NTHi isolates. Non-adherent bacteria were removed by vigorous washing of the cells and the percentage of adherent bacteria was determined by spiral plating. Values are expressed as the mean percentage $\pm$ SEM of four independent experiments of adherent bacteria relative to the starting inoculum. Although there's a tendency that strain 2 may adhere more profoundly, no statistical significant difference in adhesion between strains was detected.

Inflammatory cytokine production in human immune cells

Finally, we address the question whether there's a difference in the potency of NTHi isolates to induce the production of pro-inflammatory cytokines by human blood immune cell using a whole blood assay (WBA). We want to emphasize that in this study we deliberately chose to use blood from healthy young volunteers only. Although it might be attractive to use blood from COPD patients and/or matched healthy volunteers, here we were mainly interested in the immunogenicity of the different NTHi strains and therefore we tried to exclude as many confounders because of disease or disease severity.

Whole blood samples from 24 healthy young non-smoking volunteers were stimulated for 24 h with different inactivated NTHi suspensions and the cytokines were subsequently measured in the cell-free supernatants. Again no significant differences between the NTHi isolates were observed with respect to the stimulated release of all cytokines tested. Intriguingly, as shown in Fig. 5, a remarkable variation in cytokine release was observed. For example, TNF-α production ranged between 2000 and 30.000 pg/mL. Further analyses revealed that the prominent variability was mainly due to a large inter-individual variability between volunteers, while the intra-individual variability was rather limited. For example, Fig. 6 clearly shows that in some of the volunteers consistently a low TNF-α release was observed independent of the strain used (e.g. nr 11, 15, 16, 18 and 19), while others invariably showed a high TNF-α release (e.g. nr 1, 6, 10 and 14). Importantly, blood leucocytes counts, which potentially could explain the large inter-individual differences, were not correlated with the amount of cytokines produced (data not shown).

Post-hoc analysis including strains origin

We also retracted the isolation origin and analysed the data accordingly. NTHi isolates were obtained either during the intake procedure (stable disease, SD) or during acute exacerbation (AE). Isolates were classified as "SD" when i) detected in the sputum of the patient solely during the intake procedure and ii) the patient was clinically stable for at least 4 weeks. Alternatively, NTHi isolates were classified as "AE" only when i) isolated from the sputum obtained during an exacerbation and ii) the sputum sample of the patient was NTHi culture negative during the intake procedure. However, these selection criteria do not proof that the NTHi strain causes the exacerbation.

Next, we examined whether AE- or stable disease (SD)-associated isolates could be clustered based on their genetic profiles obtained by PFGE. Identical profiles were shared between AE-isolates obtained from different COPD patients (e.g. isolates 064508F and 058323X) implicating a genetic link among AE-isolates (Fig. 1). However, identical profiles were also observed between AE-isolates and SD-isolates from different COPD patients (e.g. isolates 069704M and 003908M). Furthermore, SD-isolates could not be categorized as distinct clusters but were randomly divided into the major clusters together with the

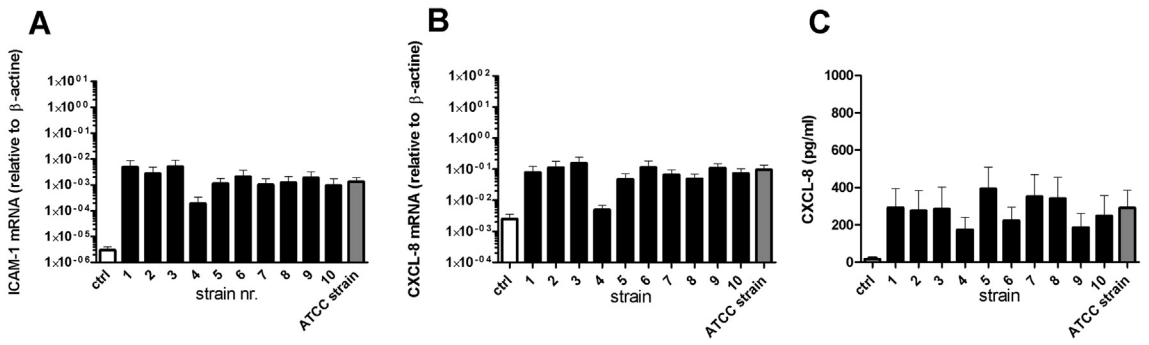

Figure 3 Relative expression of CXCL-8, ICAM-1 and production of CXCL-8 after NTHi infection by human airway epithelial cells (A549). Confluent A549 monolayers were infected with different NTHi isolates for 6 h. Values are expressed as the mean relative expression ± SEM of four separate experiments of ICAM-1 (A) or CXCL-8 (B) corrected for β-actin expression. Results revealed no significant difference between bacterial isolates. CXCL-8 production in the supernatant of A549 cells was determined with ELISA. Results show the mean CXCL-8 production (C) ± SEM in pg/mL of four independent experiments for each bacterial group. No significant difference in CXCL-8 production was detected between bacterial isolates.

AE-isolates. Therefore, the genetic profiles do not allow us to discriminate between AE-and SD-isolates. Moreover, as already shown, strains did not shown significant difference in the pro-inflammatory capacity in the *in vitro* assays indicating that AE-derived strains are not more virulent that SD-derived strains.

Discussion

Although NTHi infection has been associated frequently with the development of AE in COPD, not every infection results in an AE. To explain the different clinical outcomes after infections, we focused on the virulence of different NTHi strains, isolated from both stable COPD patients or patients experiencing an exacerbation. Although we observed significant genetic variation between the NTHi isolates analysed by PFGE, the results obtained with the *in vitro* models and the WBA did not allow us to determine whether NTHi isolates were derived from stable or exacerbating patients. Interestingly, we found marked inter-, but not intra-individual differences in immune responses between different volunteers irrespective of the NTHi isolate used.

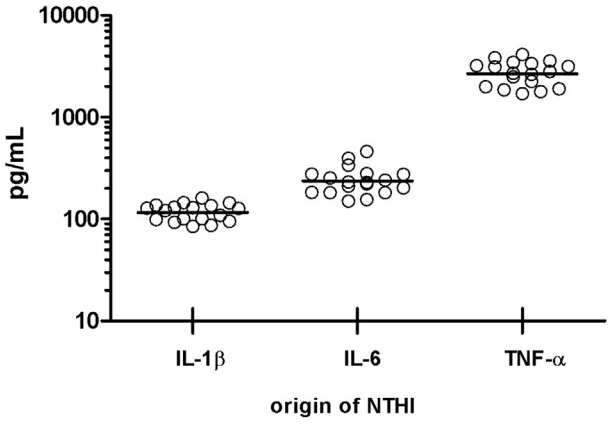

Figure 4 Pro-inflammatory cytokine response to NTHi. PMA differentiated THP-1 were stimulated with heat-inactivated NTHi isolates for 24 h. The proinflammatory cytokines (IL-1β, IL-6 and TNF-α) production in the supernatant was measured with ELISA. Results show the mean cytokine production in pg/mL of each individual NTHi isolates of four independent experiments. No significant difference between different bacterial isolates was detected.

Differential genomic content between NTHi strains has been linked to variations in bacterial virulence previously [26,27]. Significant genetic heterogeneity among NTHi isolates was also observed in the present study, which confirms earlier data by others [28,29]. Importantly, the genetic profile of the NTHi strains did not allow us to classify them as either AE- or SD-derived, as strains from the same isolation origin did not cluster in separate groups. Yet, one of the major limitations of the PFGE technique is that, although it supplies general information on the genetic diversity between different strains, it doesn't provide any specific information regarding the presence or absence of particular genes. This more specific genetic information may be relevant in terms of virulence since the expression of particular factors, e.g. specific adhesins on the bacterial surface [30] or LPS glycoforms variants [31], has been linked to NTHi pathogenesis. Moreover, strains can differ in serum resistance, nutritional factors or biofilm capacity and other virulence factors. Further identification of different NTHi strains by additional molecular genotyping is therefore required before firm conclusions regarding differences between AE and SD strains at the genetic level can be drawn.

A functional approach was used to test the capacity of the different clinical NTHi isolates to induce an inflammatory reaction in different *in vitro* assays. No significant differences between different clinical strains were observed. Interestingly, these results are in contrast with recent data from Chin and colleagues [12]. In a comparable approach they observed that AE-NTHi strains showed more efficient adhesion and induced significantly higher levels of CXCL-8 in airway epithelial cells *in vitro* than colonizing NTHi isolates. Although not entirely clear, there are some possible explanations for this discrepancy. The most important is that the current study focussed on difference in virulence among genetically distinct NTHi strains, while

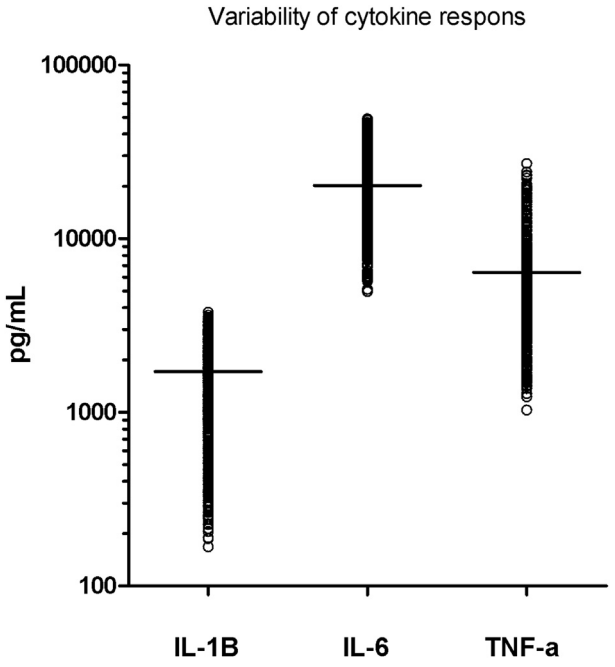

Figure 5 Whole blood cytokine response to NTHi isolates of all 24 donors. Shown are the individual cytokine responses of all donors to all NTHi isolates with its associated overall mean according to the cytokine type. Whole blood assay (WBA) was performed using blood from 24 young healthy non-smoking donors. Results revealed no difference in the amount of cytokines produced between different SD- or AE-NTHi isolates or the reference strain.

Chin et al. compared SD with AE derived NTHi isolates. The selection of SD-and AE isolates was more stringent in the Chin study as they also included the presence or absence of serum antibodies. This allowed them to more firmly correlate the appearance of a new specific strain to an AE. Unfortunately, we were unable to measure the presence of serum antibody, which is one of the limitations of the present study. Nevertheless, we only included isolates as AE strains when patients were culture negative at the time of intake, which strongly - though not definitively - indicates that the NTHi strain found during the AE was newly acquired. Furthermore, while Chin et al. used primary human

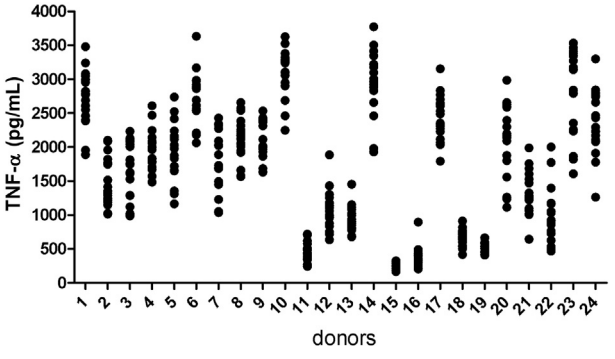

Figure 6 Variability in cytokine response of the 24 healthy donors. TNF-α cytokine responses of NTHi isolates stimulated whole blood assay from healthy volunteers ($n = 24$).

epithelial cells, in our study we used the A549 cell line. Although A549 cells are basically human alveolar epithelial cells, they were originally derived from an alveolar adenocarcinoma, and it can therefore not be excluded that they respond differently to external stimuli than primary cells. On the other hand, primary cells are most likely more heterogeneous than A549, which leaves open the possibility that differences found by Chin et al. were primarily caused by dissimilarities between primary cell cultures rather than between NTHi isolates.

Next to the lung epithelium, also pulmonary immune cells like alveolar macrophages are crucial for the host defence towards invading pathogens. To study the immune responses elicited by different NTHi strains as indices of their virulence, it would be preferable to use such primary lung immune cells. However, this requires rather invasive methods such as brochoalveolar lavage in healthy persons, which is for ethical reasons not easy to achieve. Moreover, on bacterial infection of the airways, circulating blood monocytes migrate into the sites of infection to govern the antibacterial immune response with subsequent activation of local immune effector cells [32]. In view of the latter we decided to use the monocytic THP-1 cell line to further examine the virulence of the different NTHi strains. When treated with PMA, these THP-1 cells differentiate into cells which closely resemble monocyte-derived macrophages (MDM). Since MDM represent a good alternative for tissue macrophages, such as alveolar macrophages, PMA-differentiated THP-1 cells may represent a useful model to study the inflammatory response in relation to microbial stimuli of lung macrophages [33]. However, when these differentiated THP-1 cells were treated with the distinct NTHi strains, no significant differences in the release of pro-inflammatory cytokine were observed, which is in line with the other *in vitro* results.

Yet, as the inflammatory reaction is a very complex reaction, extrapolation of *in vitro* data regarding the virulence of an infecting NTHi strain to the *in vivo* situation is difficult. To translate our *in vitro* results towards a more clinical relevant condition we used an *ex vivo* approach and we tested the inflammatory properties of the strains with a WBA [34]. A WBA was chosen because it contains important innate immune cells and therefore functions as a representative model system with limited amounts of cell culture artefacts. To our knowledge this is the first study which uses this assay to determine the virulence of different NTHi isolates. Yet, and in contrast to our expectations, within each volunteer no pronounced differences were found between the NTHi isolates regarding the capacity to provoke the release of cytokines by leucocytes in the WBA. Interestingly, a remarkable inter-individual variation of NTHi-induced cytokine responses was found among the young, healthy blood donors, despite a low intra-individual variability. To explain this pronounced inter-individual variability, several factors have been suggested, including genetic polymorphisms. Genes encoding for Toll-Like Receptors (TLR) are extremely polymorphic and many variants have been described [35]. For example some TLR4 single nucleotide polymorphism (SNPs) have been associated with hyporesponsiveness to LPS [36]. It is tempting to speculate which possible SNP are responsible for the observed pronounced inter-individual variability, however genes that

regulate this distinction are largely unknown. Despite this, robust genetic association studies showed convincing evidence between TLR SNP and a spectrum of disease, including predisposition to infections [37]. On-going investigations have to prove whether responsiveness of the (innate) immune system might be an attractive tool to predict the susceptibility for an individual patient with respect to the outcome of an infection (e.g. AE vs no AE in COPD patients).

Conclusion

The results of this study demonstrate that NTHi strains obtained from different clinical conditions (stable vs exacerbating COPD patients) do not differ significantly in terms of virulence as determined with a specific set of *in vitro* methods. Moreover, based on the genetic diversity between strains no clear association with stable disease or exacerbation was found. On the other hand, we observed a remarkable inter- but not intra-individual variability with respect to immune response to the different NTHi strains. This indicates that the individual immune reactivity might be an important predictor for the outcome of an infection of the infecting NTHi strain. And although some earlier studies in COPD patients already suggest similar large inter-individual differences between patients [38—41], future studies are required to examine whether this concept also applies to COPD patients. As the quality and magnitude of the innate immune response to bacterial infections might determine the COPD patients risk to progress to an exacerbation, a better understanding of the efficacy of an individual patient's immune system may have important clinical and therapeutic implications. Moreover, stratification of COPD patients based on their immunological responsiveness may eventually contribute to the long-lasting search for a firm concept to phenotypically characterize COPD patients [42].

Competing interest

Financial competing interest

None.

Non-financial competing interest

None.

Authors contribution

TG, GRG and GEG collected and analysed the data. TG and FS drafted and designed the study. FS, FF, GR, EW, ES, and CB critically revised the manuscript and all authors gave final approval of the manuscript.

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