Biomarkers in stable and acute exacerbations of COPD

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CHAPTER 6

6

Effects of doxycycline on local and systemic inflammation in stable COPD patients, a randomized clinical trial

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Abstract

Introduction: Neutrophilic inflammation plays a causal role in Chronic Obstructive Pulmonary Disease (COPD). Neutrophil derived myeloperoxidase(MPO) matrix metalloproteinases(MMP's), and elastases are thought to contribute to the perpetuation of the disease. The tetracycline analogue doxycycline has been shown to inhibit neutrophil-mediated inflammation. It was thus reasoned that doxycycline may attenuate neutrophil-mediated inflammation in COPD

Methods: In this double blind randomized controlled trial the effect of a 3-week course of doxycycline on sputum and systemic inflammatory parameters was evaluated in stable COPD patients. In order to exclude inflammation by bacterial colonisation patients must have 2 negative sputum cultures in the previous year. The effect of doxycycline treatment on inflammatory markers (TNF-α, IL-1β and IL-6) and neutrophil specific markers in sputum (MPO, MMP's, and IL-8) and serum C-reactive protein was evaluated. Sputum was obtained by sputum induction with hypertonic saline.

Results: A total of 41 patients were included. Ten patients were excluded as they were not able to produce sputum at the first or second visit. Baseline characteristics were similar in the two groups. In the remaining patients doxycycline did not influence sputum MPO concentrations. Also MMP-8 and 9, IL-6 and IL-8 concentrations as well as lung function parameters were not affected by doxycycline. Systemic inflammation by means of CRP was also not influenced by doxycycline.

Conclusion: A three week course of doxycycline did not influence MPO sputum levels nor any of the other inflammatory sputum and systemic markers.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by a complex neutrophil-driven inflammatory airway disease which leads to chronic airflow limitation. COPD is a growing cause of morbidity and mortality worldwide, to this day no treatment is available that can stop-or even reverse the disease process. Neutrophils play an important role in the inflammatory process and are considered a critical factor in disease progression. This is supported by the correlation that has been found between airway neutrophilia and the rate of lung function decline and the finding that airway neutrophilia is related to small airway dysfunction.[1;2] Furthermore associations are found between COPD and markers of neutrophilic inflammation such as myeloperoxidase (MPO), matrix metalloproteinases (MMPs) and elastase.[1;3;4]

It has been shown that a sustained neutrophilic response contributes to the disease process indirectly through perpetuation of the inflammatory response, and directly through contributing to the airway remodelling.[5] This is exemplified by degradation of extracellular matrix (ECM).[6;7] This degradation results in the destruction of alveoli and airway remodelling which is a hallmark of moderate to severe COPD.[8] These observations characterize the neutrophilic inflammatory cascade as a candidate target for COPD.

Independent of the antibiotic properties, tetracyclines show comprehensive anti-inflammatory effects which include a strong effect on neutrophilic mediators, neutrophil and cytotoxic T cell recruitment.[9;10] A tetracycline analogue, doxycycline, markedly reduced airway inflammation in an animal experiment.[11;12] Moreover, a recent randomized clinical trial of doxycycline in patients with COPD showed clear reductions of C-reactive protein plasma levels (CRP) as well as improved lung function.[13] A critical question arises whether these positive effects observed were based upon a reduction of bacterial load or due to a direct anti-inflammatory effect. In the current proof of concept study we aimed to assess the anti-inflammatory effects of doxycycline on sputum inflammatory markers in COPD patients. To that end we performed a placebo controlled randomized clinical trial of doxycycline in patients without airway bacterial colonization during the last year.

Material and Methods

Study subjects

The study was conducted in the outpatient population of the department of pulmonary diseases of the Medical Centre Alkmaar, Alkmaar, and the Netherlands. The study protocol was approved by the local ethics committee and carried out in accordance with good clinical practice. The trial was registered at clinicaltrials.gov (NTC00857038).

Written informed consent was obtained from all the participants. The study was carried out between August 2009 and December 2010.

The present study is a randomized double blinded placebo-controlled study, designed to investigate the efficacy of doxycycline in addition to standard treatment in patients with stable COPD GOLD II-III.[14] Randomization was based on a one-on-one allocation by means of pre-numbered containers containing identical looking capsules with placebo or doxycycline. The allocation sequence was computer generated and was kept in a safe at the hospital pharmacy throughout the course of the study. Patients included were, age over 40 years and without airway bacterial colonization, defined as two negative sputum cultures or broncho-alveolar lavage cultures in the previous year. Exclusion criteria included respiratory diseases other than COPD, use of systemic corticosteroids or other immunosuppressive drugs within one month prior to inclusion in the study and allergy for tetracyclines or a history of substantial side effects. Subjects with an acute exacerbation as defined by Anthonisen et al, or an active respiratory or non-respiratory infection one month prior to the study were also excluded.[15] The primary end point was change in sputum myeloperoxidase (MPO) levels from baseline to end of 3 weeks. MPO was chosen because it is a well-known marker of neutrophil presence and activation.[16] Secondary endpoints were changes in sputum interleukin (IL)-6 and IL-8, as markers of inflammation and recruitment of neutrophils, sputum MMP-8 and MMP-9, as additional markers of neutrophil activation, sputum granzyme A as a marker of cytotoxic $CD8^+$/NK-cell activation, sputum TNF-α and IL-1β as markers of macrophage function, serum C-reactive protein (CRP) as a marker of systemic inflammation and lung function from baseline to end of 3 week treatment. Treatment dose and duration of 3 weeks was chosen based upon the trials of Lindeman et al, one week of treatment was added to ensure maximum effect because of the small sample size of the current study.[17;18]

At initial screening visit patients were asked to discontinue their inhalation corticosteroids if they used any. After this run-out period of 4 weeks, baseline testing and sputum induction were performed. Subsequently patients were randomized and treated with doxycycline 100mg/day or placebo for 21 days. Hundred milligram of doxycycline was chosen based on earlier research and availability of doxycycline 100mg as well as matching placebos. In the event of an acute exacerbation of COPD or other indication for use of steroids or antibiotics, patients were excluded from the study. Patients were also excluded if sputum induction was unsuccessful at the start or end of the study.

Sputum induction and analyses

Sputum induction was performed by inhalation of hypertonic saline 3% for 10 minutes. Saline concentrations were increased to 4% and 5% at intervals of 10 minutes, in

accordance with a protocol described before.[2] If patients were able to produce sputum spontaneously, sputum induction proceeded as planned, spontaneous produced sputum was not analysed to minimize bias. Induced sputum was processed by the whole-sample technique using dithiothreitol (DTT).[19] Total cell numbers were determined by counting manually in a Bürker counting chamber. Samples containing less than 50% viable cells, as assessed by Trypan blue dye exclusion, were excluded from analysis. The remaining samples were centrifuged for 5 minutes at 700*g*. Cells were then separated from the supernatant and cytocentrifuged at 500rpm for 2 minutes, dried and stained with Romanovski and Jenner-Giemsa. Squamous and non-squamous, macrophages, lymphocytes, neutrophils and eosinophils were identified. Differential counts are expressed as a percentage of non-squamous cells. For differential cell counts 200 non-squamous cells were enumerated in samples with more than 10% eosinophils. The number of non-squamous cells is increased to 500 or 1000 cells if the percentage of eosinophils is between 1% and 10% or less than 1% respectively. Sputum samples containing more than 75% squamous cells on differential cell counting were excluded from cell differential analysis. Supernatants were re-centrifuged and stored in aliquots at -80˚C until analysis.

MPO was measured by ELISA (DY3174; R&D Systems, Abingdon, UK)in sputum supernatant with lower limits of detection of 1 ng/ml being 2-fold the background absorbance.[20] Granzym A was determined with a PeliKine ELISA following the supplier's recommendations (Sanquin, Amsterdam, the Netherlands) lower limit of detection 41.25 pg/ml. IL-6, IL-8, IL-1β and TNF-α were determined by Luminex (BioRad reagents, Veenendaal, the Netherlands) lower limit of detection 0.12pg/ml, 0.093pg/ml, 0.5pg/ml and 0.28pg/ml respectively. Samples were measured in one assay run to limit variation. In all assays a bias by the sulphur-bridge reducing reagent DTT was ruled out based upon appropriate controls. To that end we showed for all parameters that a 1 in 50 dilution is required to dilute out effects of DTT. At dilutions ≥1 in 50 dilution the samples diluted out properly. Spike recoveries were between 80-100% for most.

MMP-8 and MMP-9 activity assays (Amersham Biosciences; Buckinghamshire, UK) were performed according to the recommendations of the supplier. These assays measure both active (mature) MMP as well as total MMP (already active plus activatable [*ie*, latent] pro-MMP) activity, but are insensitive to proteinase inhibitor complexes. In brief, MMP-8 or MMP-9, is captured by a specific antibody that has been immobilized on a microtiter plate. The amount of active MMP is measured directly by the incubation of the captured MMP with modified pro-urokinase (Ukcol), and subsequent activation of Ukcol is quantified by a chromogenic substrate (S-2444). Colour development is recorded at 405 nm at different time intervals. Total MMP activity (*i.e.*, the pro-MMP and active MMP forms) is assessed through the activation of pro-MMP by pre-incubation with 0.5 mmol/L p-aminophenylmercuric acetate for 2 h at 37°C before the addition of modified Ukcol and chromogenic substrate. Activity is expressed in

recombinant enzyme equivalents (recEEs) in nanograms per mL. The lower detection limits were 5.0 ng/mL recEE for MMP-8 and 2.7 ng/mL recEE for MMP-9.
DTT may interfere with MMP activity. In order to study the effect of DTT on MMP activity, standard curves of appropriate recombinant proteins were incubated with 0.1% DTT (Sputolysin) for 20 min at room temperature under the same conditions as sputum samples during processing. DTT concentration and incubation time was based on an earlier optimization procedure.[4] An incubation time longer than 20 minutes did not result in a higher recovery but may interfere with MMP activity. In order to study the effect of DTT on MMP activity, standard curves of appropriate recombinant proteins were incubated with 0.1% DTT (sputolysin) for 20 minutes at room temperature under the same conditions as sputum samples during processing. The presence of DTT did not affect MMP activities, indicating that DTT had no effect on the immunocapture activity assays used in this study (data not shown).

Statistical analysis

As being a proof of concept study, sample size was based upon a pilot trial design.[21] We decided to include 12 evaluable patients in each arm. Statistical evaluations were accomplished with Mann-Whitney test for difference in change amongst both groups in continuous variables and Wilcoxon test for difference in change within groups in continuous variables. Chi square test was used for dichotomous variables. Possible associations between sputum MPO, IL-6, IL-8 and cellular content of sputum were evaluated by Spearman's correlation. A p-value <0.05 was considered significant. SPSS 20.0 for PC (IBM) was used for the statistical analyses.

Results

One hundred and eleven patients with COPD were screened. Fifty-four patients refused to participate and 11 patients did not meet the inclusion criteria. One patient had an exacerbation during the run-in phase, and four patients had worsening of symptoms after

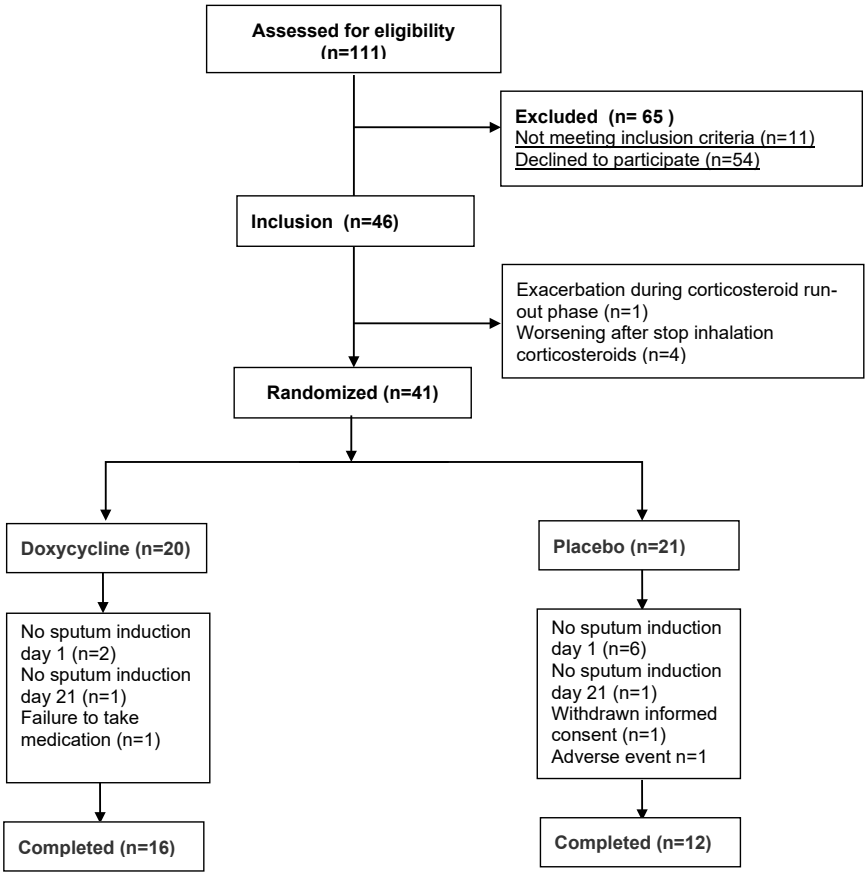

Figure 1 trial profile

Hence, 41 patients were randomized. In the doxycycline group two patients at day 1, and 1 patient at day 21 failed to produce sputum. In the placebo group six patients at day 1 and one patient at day 21 were not able to produce sputum after induction (p=0.108). One patient withdrew consent and one patient was excluded because of systemic corticosteroid because of non-bacterial arm inflammation in the placebo group, and one person failed to use his medication in the doxycycline group. One adverse event was reported in the placebo group. No adverse events were reported in the doxycycline group. The adverse event reported was non-bacterial inflammation of the arm and treated with systemic corticosteroids. Therefore study medication was discontinued.
The groups were similar with respect to age, gender, smoking history, use of inhaled corticosteroids, baseline sputum inflammatory markers, baseline cellular sputum components and lung function parameters although differences were found for baseline in FVC (Table 1).

Table 1: Baseline characteristics

	Placebo n=21	Doxycycline n=20	p-value
Age (years)	70 (63-75)	67 (63-72)	0.620
Male gender (n, %)	18 (85.7)	16 (84.2)	0.894
BMI (kg/m2)	26.5(24.7-29.7)	25.5(24.1-27.5)	0.303
FEV1 (L)	1.63(1.39-2.08)	1.88(1.64-2.17)	0.246
$FEV1\%_{pred}$	59(54-70)	60(52-66)	0.979
FVC (L)	2.94(2.70-3.35)	3.53(3.08-4.15)	0.023*
$FVC\%_{pred}$	75(69-87)	88(77-104)	0.055
FEV1/FVC ratio	55.6(43.4-63.0)	54.6(46.7-63.5)	0.732
Current smokers (N, %)	4 (19)	8 (40)	0.141
Pack years (N)	32(20-40)	37(24-46)	0.329
Number of exacerbations last year (n)	0(0-1)	0(0-1)	0.663
ICS usage (N, %)	11(52.4)	8(40)	0.473
SABA treatment (n, %)	6(28.6)	4(20.0)	0.595
LABA treatment (n, %)	11(52.4)	12(62.0)	0.823
SAMA treatment (n, %)	1(4.8)	0(0)	0.240
LAMA treatment (n, %)	12(57.1)	11(55.0)	0.890

All data are represented as median (IQR) unless specified otherwise
BMI: body mass index, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity. ICS: inhalation corticosteroids, SABA: Short-acting beta agonist, LABA: Long-acting beta agonist, SAMA: Short-acting muscarinic antagonist, LAMA: Long-acting muscarinic antagonist

Doxycycline as compared to placebo did not influence sputum MPO, IL-6 and IL-8 levels as well as MMP-8 and 9 activity at day 21 (Table 2 and Figure 2).

Table 2: Sputum biomarkers

	Placebo (n=12)			**Doxycycline (n=16)**		
	T= day 0	T= day 21	P-value	T= day 0	T= day 21	P-value
MPO (µg/ml)	9.2 (2.1-100.8)	6.9 (4.8-36.6)	0.768	6.1 (4.4-27.9)	6.4 (2.2-21.3)	0.910
IL-6 (pg/ml)	10.5 (0.1-226.7)	38.7 (0.9-232.9)	0.767	16.0 (4.8-102.8)	12.7 (1,0-62.9)	0.570
IL-8 (pg/ml)	2155 (521-4435)	1554 (543-10750)	0.718	797 (297-2945)	1268 (284-4033)	0.880
MMP-9 (mg/ml)	67.8 (26.9-198.7)	53.2 (10.8-108.8)	0.488	40.3 (8.4-146.7)	29.7 (7.7-96.7)	0.520
active MMP-9 (mg/ml)	7.0 (5.2-13.6)	6.8 (1.5-9.9)	0.326	2.8 (2.0-13.0)	3.1 (1.7-5.7)	0.631
MMP-8 (mg/ml)	247.6 (185.6-458.1)	265.7 (151.7-397.6)	0.488	275.9 (147.2-443.9)	264.2 (163.3-323.9)	0.468
active MMP-8 (mg/ml)	256.8 (147.9-518.9)	289.4 (151.2-422.6)	0.954	241.1 (123.2-395.9)	201.7 (102.7-348.9)	0.548

All data are represented as median (IQR).
Wilcoxon test for difference in change within groups in continuous variables was used.

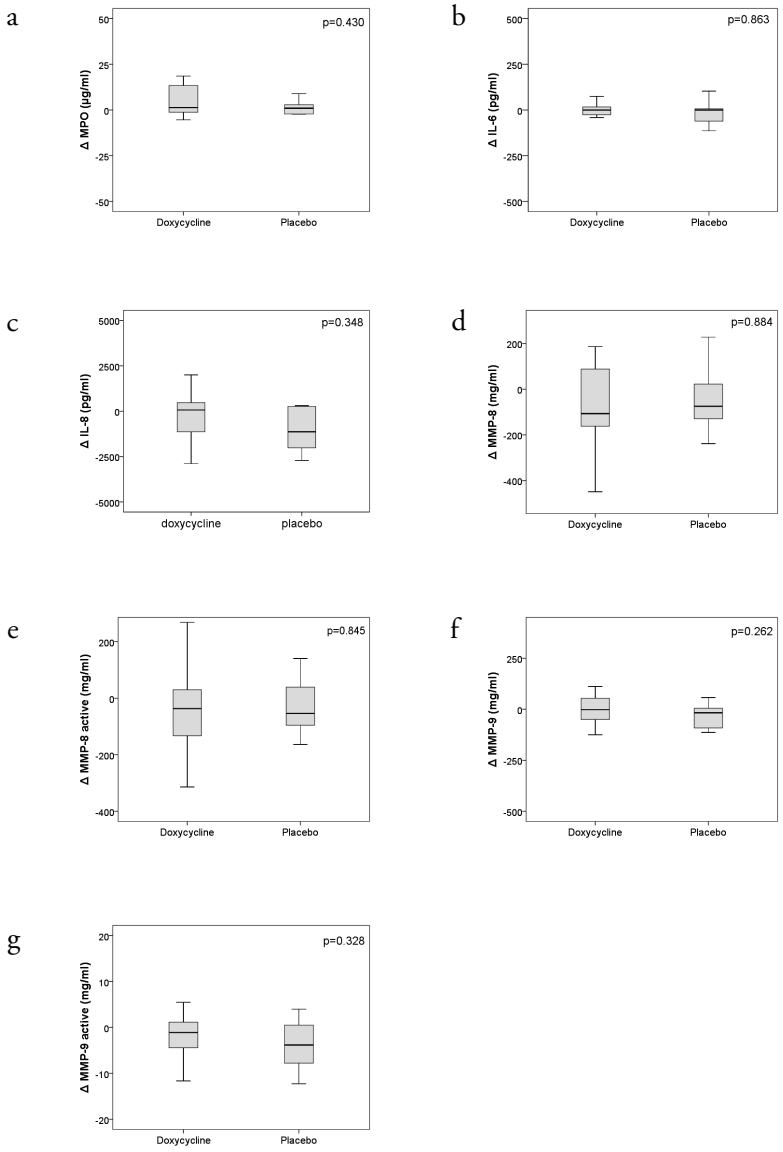

Figure 2: a) Delta sputum MPO concentration (day 0-21), b) delta sputum IL-6 concentration (day 0-21), c) delta sputum IL-8 concentration (day 0-21), d) delta sputum MMP-8 concentration (day 0-21), e) delta sputum MMP-8 active concentration (day 0-21), f) delta sputum MMP-9concentration (day 0-21), g) delta sputum MMP-9 active concentration (day 0-21)

Biomarker levels were similar in the doxycycline and placebo group at baseline as well as at day 21(Table 3). TNF-α, IL-1β and Granzym-A were all below the detection threshold of the assay for both time points. Doxycycline did not influence systemic inflammation represented by serum CRP, numbers of peripheral blood neutrophils or total leucocyte count (Table 3).

Table 3: Cellular components in sputum blood and serum CRP

	Placebo(n=12)			**Doxycycline(n=16)**		
	T=0	T=21 days	P-value	T=0 days	T=21 days	P-value
Total sputum cell count	15.5	12.1		17.8	13.3	
($x10^6$/ml)	(8.51-22.44)	(9.8-18.3)	0.705	(12.0-25.1)	(7.6-17.0)	0.108
Sputum neutrophil cell count	9.0	7.6		9.4	6.1	
($x10^6$/ml)	(4.5-14.2)	(3.4-11.4)	0.545	(5.1-18.4)	(3.1-10.0)	0.198
Relative sputum neutrophil	56.8	55.4		65.3	56.3	
count (%)	(37.0-69.3)	(46.0-61.9)	0.762	(37.7-74.5)	(35.9-62.0)	0.358
Total white blood cell count	7.3	7.1		7.2	6.6	
($x10^9$/ml)	(6.1-8.5)	(6.1-8.4)	0.945	(6.1-7.8)	(5.3-7.7)	0.241
Total blood neutrophil count	4.1	4.1		4.5	4.0	
($x10^9$/ml)	(3.5-5.0)	(3.3-4.6)	0.982	(3.7-4.8)	(3.4-4.6)	0.418
Relative blood neutrophil count	57.8	56.0		58.7	56.9	
(%)	(53.5-64.0)	(52.7-59.6)	0.890	(51.3-60.8)	(50.3-65.8)	0.705
Serum CRP (mg/L)	4.40	4.0		4.3	2.90	
	(2.80-5.70)	(2.40-6.60)	0.854	(1.70-6.30)	(1.80-4.40)	0.380

All data are represented as median (IQR), Wilcoxon test for difference in change within groups in continuous variables was used. CRP: C-reactive protein

Clear correlations were found between the number of neutrophils in sputum and sputum MPO at the start of the study (r=0.553 [p=0.005]) and at day 21 (r=0.544 [p=0.006]). Doxycycline therapy did not improve lung function parameters. Median change in FEV1 (L) in doxycycline group was -0.11 L (IQR 0.29-0.9L) whereas placebo had a median decrease of -0.3L (IQR -0.12-0.5L) [p=0.623]. Median change in FEV1 as percentage of predicted neither differed between doxycycline -4 %(IQR -10.0-3.0%) and placebo -1 %(IQR -4.0-2.0%) [p= 0.812]. FVC in litres as well as in percentage of predicted did not change within both groups, doxycycline -0.09L (IQR -0.32-0.1L) vs placebo -0.07L (IQR -0.19-0.14L) [p= 0.657], respectively, and doxycycline -2 %(IQR -8-3%) vs placebo -2%(-4.0-5.0%) [p= 0.623].

Discussion

COPD is a complex disease involving many types of immune responses that recruit many types of innate and adaptive immune cells, yet it is predominantly recognized as a neutrophilic inflammatory disorder.[3;22] MPO is a well-known marker of neutrophil presence as well as activation.[23;24] It is a heme-containing peroxidase expressed abundantly in neutrophils and to a lesser extent in monocytes. MPO is one of the principal enzymes released from secondary granules following neutrophil activation.[24] Although the generation of oxidants by MPO is beneficial in terms of the immune response to invading pathogens, there is considerable evidence that inappropriate stimulation of oxidant formation can result in host tissue damage.[25] Induced sputum represents the cellular pattern of the mucosa of the lower airways, and analysis of this sputum composition makes non-invasive measurement of airway inflammation possible[1]. Therefore, sputum MPO may be a potential non-invasive biomarker that reflects the severity or prognosis of COPD. A potential novel way to oppose this neutrophilic inflammation is doxycycline.[17;18;26] Doxycycline, besides its antibiotic properties has been shown to interfere with aspects of neutrophil-mediated inflammation as well as through inhibition of MMP's.[27] Although these anti-inflammatory effects of doxycycline never has been described in respiratory diseases, these effects where described in other chronic diseases such as abdominal aneurysm (AA) and periodontitis.[17;18,28] Several parallels exist between AA, periodontitis and COPD.[29;30] Both with regard to risk factors as well as to the underlying pathophysiology in which persistent inflammation and excess matrix turnover play a pivotal role.[29] It was therefore thought that doxycycline might reduce chronic inflammation in COPD. Therefore we designed this randomized controlled clinical trial that specifically investigates the anti-inflammatory effect of doxycycline on sputum inflammatory markers in stable patients with COPD without bacteria in their sputum. MPO, our primary outcome was not influenced by doxycycline treatment.

These findings are in line with an earlier trial which showed no effect of 100mg of doxycycline a day on MPO levels in patients with previous coronary artery surgery.[31] However, another study showed a decrease of MPO levels in obese patients with type 2 diabetes mellitus.[32] Although none of these trials studied the specific effect of doxycycline on MPO levels in sputum of stable patients with COPD, a possible explanation for our results might be that our population was specifically selected to be not colonized by PPM's. This is further emphasized by the relative low levels of MPO at baseline compared to other studies.[1] As is known levels of MPO as well as other pro-inflammatory cytokines are increased by higher bacterial load in sputum.[20] Another explanation could be the length of antibiotic treatment. Patients were only treated for 3 weeks, yet this seems not likely as another trial only treated patients with doxycycline for 10 days and saw a significant reduction in MPO levels in nasal secretions after 20 days of doxycycline using similar dosage as in our trial.[33]

Our data showed no effect of doxycycline on neutrophil content as well as on the amount of MMP8 and MMP9 in sputum. This is in contrast with earlier research in which doxycycline dose independently reduced MMP8 and MMP9 due to a reduced neutrophil content of the aneurysmatic wall of the aorta after two weeks of doxycycline (50, 100 or 300 mg once a day) in patients who underwent elective replacement surgery of the abdominal aneurysmatic aorta.[17;18]This difference in local inflammation by MMP-8 and MMP-9 can be explained by the low cell count as well as a low percentage of neutrophils in the induced sputum at the beginning of study compared to previous trials.[1;20] This might be due to the absence of bacterial colonization in our patient group. Another possibility is that doxycycline has a different mode of action in the lung compared to the aorta or periodontal disease.

IL-6 plays an important role in the progression of COPD. It is known that patients with stable COPD have higher plasma levels of IL-6 compared to healthy volunteers and levels of circulating IL-6 have also been shown to be associated with lung function impairment.[34] Tetracyclines are known inhibitors of IL-6.[35] In our trial IL-6 levels were not affected by doxycycline. This might be due to the relative low levels of IL-6 at the start of the study.[36] As is known IL-6 levels correlate to bacterial load in sputum.[37] Therefore our hypothesis is that doxycycline does not have an anti-inflammatory effect on IL-6 levels, yet the low levels observed before and after treatment with doxycycline are caused by the absence of bacteria. Yet this does not explain the effects of doxycycline observed in earlier research.[35]

In our trial we did not observe effect of doxycycline on lung function parameters nor on systemic inflammation. This is in contrast with earlier results shown by Dalvi et al.[13] These differences found may be explained by the shorter treatment period and the fact that they did not exclude patients with PPM in sputum, and that therefore the observed effect is antibacterial, this might also explain the differences found in lung function.

Modulation of inflammation in COPD might be one of the future keystones in the treatment of COPD. Although in our study we were not able to show effect of doxycycline on inflammation, other possible candidate to modulate inflammation in COPD are the group of macrolides, a group of broad spectrum antibiotics which has been shown to have significant immunomodulatory effects related to the macrolytic lactone ring.[38] Extensive research has been done with azithromycin showing a reduction in pro-inflammatory interleukins and TNF-α resulting in a decreased number of exacerbations and an increased time to next exacerbation.[39;40]

Shortcomings of this study are the small sample size and a high percentage of lost to follow-up due to the fact that subjects could not evacuate sputum during sputum induction. This is a strong argument that fits the assumption that in our trial we are looking at a distinct phenotype of COPD in which there is little bacterial inflammation and mucus hypersecretion. Although there is a certain amount of inflammation present, this is not influenced by doxycycline. Another shortcoming of this study is the absence

of a sputum culture at the start of the study. However earlier research showed that the amount of inflammation correlates well with the presence of bacteria.[4;41] This combined with the fact that at baseline low inflammation levels in sputum were present and the absence of PPM's in sputum cultures in the prior year, advocates the fact that our subjects were not colonized by PPM's.

This is the first placebo controlled randomized clinical trial that investigates anti-inflammatory effect of doxycycline in COPD patients. Strength of this study are the good correlation of the individual biomarkers as well as the methodology. Sputum was induced by the same study nurse at the same time of the day, so fluctuations of sputum quality throughout the day were excluded. A weakness of this study is the pilot design. Due to heterogeneity of the population regarding types of inflammation in COPD a higher number of patients might be needed to draw a more firm conclusion regarding doxycycline being an anti-inflammatory drug in COPD.

However in this study we were not able to show effect of a 3 week course of doxycycline on sputum MPO, or on any of the other sputum and serum inflammatory markers as well as lung function parameters in stable COPD patients without airway bacterial colonization. In spite of current findings, it might be possible that the tetracycline analogue doxycycline is able to attenuate inflammation in COPD patients with a latent infection or a more outspoken pro-inflammatory phenotype. On the basis of the current study we cannot recommend doxycycline as an anti-inflammatory agent for patients with stable COPD.

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