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Impact of acute exposure to tobacco smoke on gelatinases in the bronchoalveolar space

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Short title: BAL gelatinases and tobacco smoke

ABSTRACT

Clinical studies have indicated an increased gelatinase activity in the airways of patients suffering from COPD caused by tobacco smoke. This study aimed to determine whether acute exposure to tobacco smoke *per se* causes a substantial and lasting impact on gelatinases and their inhibitors in the peripheral airways of atopic and non-atopic human subjects. Bronchoscopy with bronchoalveolar lavage was performed on occasional smokers with and without atopy before and after smoking 10 cigarettes over a 48 h period. Samples from a group of never-smokers not exposed to tobacco smoke served as controls. Gelatinase identity and activity were measured using zymography and gelatinase activity assay and concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 were measured using ELISA. The results revealed no pronounced changes in identity, net activity or concentration of the gelatinases or in concentrations of TIMP-1 and TIMP-2 in bronchoalveolar lavage fluid before and after acute exposure to tobacco smoke. In conclusion, this experimental study indicates that acute exposure to tobacco smoke does not cause any substantial impact on gelatinases or their inhibitors in the peripheral airways, irrespectively of atopy status; a finding that is compatible with the fact that it takes many years of tobacco smoking to establish COPD.

INTRODUCTION

In the lungs and in other organs, several matrix metalloproteinases (MMPs) are believed to be important for maintaining normal turnover of extra cellular matrix and these MMPs can be divided functionally into several groups, including gelatinases, collagenases and membrane-type of MMPs, depending on what specific molecules they degrade (1). The gelatinases include MMP-2 and MMP-9, which apart from gelatine, also degrade collagen, elastine, fibronectine as well as other extra cellular matrix proteins (2). MMP-2 and MMP-9 have specific inhibitors; tissue inhibitors of matrix metalloproteinase (TIMP)-2 and TIMP-1 (1). As a consequence, the endogenous control of TIMP production might be as important as the control of MMPs, to maintain gelatinase homeostasis.

Interestingly, previous clinical studies indicate an altered production of both gelatinolytic MMPs and their matching TIMPs in chronic lung disease caused by tobacco smoke. Thus, an up-regulation of MMP-9 and MMP-2 has been demonstrated in the lungs of patients with manifest chronic obstructive pulmonary disease (COPD) and an up-regulation of MMP-9 has been detected in the lungs of patients with emphysema (3). This up-regulation of MMPs, especially if occurring in combination with a down-regulation of TIMPs, may lead to an imbalance in the gelatinase homeostasis (4, 5). However, the course and order of proteolytic events preceding manifest COPD in human tobacco smokers remain largely unknown. As a consequence, it is still uncertain to what extent acute exposure to tobacco smoke *per se* leads to an imbalance in the gelatinase homeostasis in the peripheral airways of clinically healthy humans. One study on induced sputum from occasional smokers have shed some light by forwarding evidence that, in the proximal airways, acute exposure to tobacco smoke does cause a transient increase in the number of neutrophils; an inflammatory cell that constitutes a

potentially important source of the gelatinase MMP-9 (6). However, the referred study on sputum did not indicate any differences in MMP-9 or TIMP-1 in the proximal airways after smoke exposure. Moreover, apart from reflecting the proximal and not the peripheral airways , the sputum technique is problematic in that it does induce neutrophil accumulation *per se*, if used repeatedly (7). Induced sputum thus introduces a confounding factor already at baseline, since it may cause even more neutrophils to become exposed to the locally accumulated stimuli associated with exposure to tobacco smoke. In addition, sputum contents are likely to mainly reflect proximal and not the peripheral airways, as opposed to bronchoalveolar lavage (BAL) fluid (8-10).

Yet another issue of clinical interest in the context of COPD is the importance of atopy for the gelatinase homeostasis in human lungs. Previous studies have demonstrated an increased MMP-9 activity in the airways of patients with asthma but the impact of acute exposure to tobacco smoke has not previously been addressed in the peripheral airways of atopic subjects (11).

The current study was designed to determine whether an acute exposure to tobacco smoke exerts a substantial and lasting impact on gelatinases and their inhibitors in the lower airways of clinically healthy human subjects. The study also addressed the potential importance of atopy in this specific context. Specifically, in addition to assessing the gelatinase/gelatinase inhibitor ratio in terms of concentrations of MMP-2 and -9 and TIMP-2 and -1, our study ascertained the identity and the net activity of the dominant gelatinases in the peripheral airways and controlled for the examination procedure (i.e. bronchoalveolar lavage) *per se.*

MATERIAL AND METHODS

Study subjects

Three groups of study subjects with normal lung function were recruited for this study; non-atopic occasional smokers, atopic occasional smokers and never-smokers. All subjects gave their written informed consent to participate in the study which was approved by the Ethics committee in Göteborg. All three groups had been free from smoking and respiratory infections at least four weeks prior to participating in the study. Spirometry was performed to measure and confirm normal lung function in each individual. All atopic occasional smokers in our study had a history of subjective symptoms from the upper and/or lower airways. The history of atopy was objectively confirmed in the through Phadiatop^{TM} testing of specific IgE and by assessing total IgE-levels (Phadia AB, Uppsala, Sweden) in blood. All subjects underwent two bronchoscopies including bronchoalveolar lavage; the first at day 1 termed BAL1 and a second at day 14 termed BAL2. On day 12 and 13, all occasional smokers smoked in total 10 filter cigarettes of a commercial brand (tar 10 mg, nicotine 0,8 mg) that were purchased commercially (no gifts). To be considered an occasional smoker, the subjects had to habitually smoke cigarettes at least at one occasion per month and maximum at four occasions per month. The dose (number) of cigarettes was chosen based upon the clinical observation that none of the recruited occasional smokers habitually smoked more than 20 cigarettes over a 48 hour period. We reasoned that it would be unethical to exceed the number of cigarettes the recruited subjects would habitually smoke by average, and therefore we chose a dose of 10 cigarettes over 48 hours and considered this ethically impregnable. The smoking status for each subject was controlled by measuring the urine cotinine level at the time of each of the two bronchoscopies. To be included, all subjects had to display cotinine levels below 100ng/ml prior to the first bronchoscopy. For the continued inclusion of occasional smokers at the time of the second bronchoscopy (i.e. as a confirmation of the

intervention smoke exposure), these subjects had to display cotinine levels at least five-fold those at the time of the first bronchoscopy. Subjects were excluded if they suffered from any infection between the two bronchoscopies.

Twenty-nine occasional smokers were recruited and underwent the two bronchoscopies. Seven of these were later excluded from further analysis since they did not meet our criteria for cotinine levels in urine. Of the remaining 22 occasional smokers, 13 were non-atopic and 9 were atopic. Eighteen never-smokers were recruited, out of which three were subsequently excluded due to infections during the study. The patient characteristics of the 13 non-atopic occasional smokers, 9 atopic occasional smokers and 15 never-smokers are shown in Table 1. Age and lung function did not differ markedly between the groups. Cotinine levels in urine at the time of bronchoscopy 1 and bronchoscopy 2 are shown in Table 2 in Supplement 1.

Table 1 Patient characteristics, median and range

	Never-smokers	Occasional Smokers	Atopic occasional smokers
n	15	13	9
Age	23 (21-36)	26 (22-44)	23 (21-26)
Sex female/male	8/7	7/6	5/4
FEV_1% of pred	108 (96-129)	105 (95-130)	107 (85-125)
FEV_1/FVC	89 (74-99)	85 (75-99)	91 (83-99)

Bronchoscopy, BAL sampling and handling

Bronchoscopies were performed according to standard procedure using a flexible bronchoscope. Bronchoalveolar lavage was performed in the right middle lobe, utilising instillation of totally three times 50 ml of phosphate-buffered saline (PBS). A first portion of 20 ml PBS was instilled as a bronchial wash and collected separately to avoid contamination

from the proximal airways in the BAL. BAL fluid was collected in polypropylene tube and kept on ice until reaching the lab. Total cell count and trypan blue exclusion on the BAL cells was made and the BAL samples were centrifuged to separate cells from BAL fluid. Cells were resuspended in buffer and cytospins made for May-Grünwald-Giemsa staining and subsequent differential counting of 600 cells per sample according to standard morphological criteria. The BAL fluid was aliquoted and kept in -80^{o}C until further analysis.

Gelatinases and gelatinase inhibitors in BAL fluid

Identity of gelatinases

Zymography was used to identify MMP-2 and MMP-9 bands and to screen for total MMP-2 and MMP-9 activity in the BAL fluid of the 5 first subjects in each study group. Since the process of zymography may physically disassociate bound TIMPs from gelatinases, we utilized representative bands for densitometry (Kodak-1D image analysis software) as a quantitative assessment of the total gelatinase activity. Identical areas around individual bands were scanned and a background subtracted net intensity value was generated. The assay was performed as described previously (12). Briefly, gelatin (2 mg/ml, Labchem, Pittsburgh, USA) was added to a 10% SDS gel before casting. Under non-reducing conditions 10 µl of neat BAL samples were run through the gel at a constant voltage (200V). After the electrophoresis the gel was washed twice in 2.5% Triton X-100, incubated at 37^{o}C overnight in zymography buffer (50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 0.01% NaN_3) and subsequently stained with Brilliant Blue (B7920 Sigma-Aldrich, Saint Louis, USA).

Quantity of gelatinases and gelatinase inhibitors

The concentrations of MMP-2 (pro- plus active forms), MMP-9 (pro- plus active forms), TIMP-1 and TIMP-2 protein were determined in all of the BAL fluid samples using commercial ELISA kits from R&D systems (Abingdon, UK) and run according to the manufacture's recommendations. For MMP-2 analysis BAL samples were concentrated, as required for detection (15- to 30-fold), prior to ELISA. Amicon Ultra tubes with 5 kDa cut off were used (Millipore, Billerica, USA) for the concentration procedure. According to the manufacturer of the ELISA kits, the sensitivity of the ELISAs were 0.16 ng/ml for MMP-2, 0.156 ng/ml for MMP-9, 0.08 ng/ml for TIMP-1 and, finally, 0.011 ng/ml for TIMP-2.

Net activity of gelatinases

The net gelatinase activity was measured in all of the BAL fluid samples using a fluorescence-conjugated gelatine substrate (D-12054 DQ gelatin from pigskin, Invitrogen, Mount Waverley, Australia). BAL fluid proteins were precipitated using acetone (2 ml BAL fluid in 8 ml ice cold acetone), the pellet was air dried and then re-suspended in 200 μl PBS, effectively concentrating the BAL fluid ten-fold. The concentrated BAL fluid (25 μl) was incubated with the gelatine substrate (5 μg) diluted in 175 μl gelatinase substrate (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% NaN_3 for 16 hours, gently rotating in a 37^{o}C incubator. PBS alone was used for background readings that were subtracted from all samples measurements. The fluorescence intensity of the digested substrate was then measured in a FlexStation II micro plate reader (Molecular Devices Corporation, Sunnyvale, USA) at the absorption/emission wavelength of 495/515 nm.

Statistics

Kruskal-Wallis was used to detect differences between the three study groups. In case the test showed a statistical significant difference between the groups then Mann Whitney was used

for testing which of the three groups that differed from each other. Wilcoxon Signed Ranks Test was used to test differences between the paired "BAL1" and "BAL2" samples within each study group. Differences were considered statistically significant if the p-value was less than 0.05. Correlation analysis was performed by calculating Spearman's rank correlation coefficient. SPSS software (Chicago, USA) was utilised to perform the statistic calculations.

RESULTS

BAL cell counts

BAL recovery, total cell count and cell viability did not differ markedly between BAL1 and BAL2 in any of the study groups or between the study groups (Table 3). Likewise, the differential counts revealed no substantial differences in percent of neutrophils or macrophages, two important producers of the investigated MMPs. Neither were any differences found in the percentage of lymphocytes. The percentage of eosinophils, however, was higher in BAL2 than in BAL1 among never-smokers ($p=0.016$) and atopic smokers ($p=0.039$) (Table 3 in Supplement 1). A similar trend was observed in non-atopic smokers but this trend did not prove statistically significant.

Gelatinases and gelatinase inhibitors in BAL fluid

Identity of gelatinases

The identification of the dominant gelatinases in BAL fluid was ascertained by utilizing zymography with gelatine as the substrate. BAL samples from the first five subjects in each study group were studied. We identified three main bands, approximately 70, 90 and 150kDa in size (Figure 1). Based on their respective molecular weights the 70 and 90kDa bands were

identified as pro-MMP-2 and pro-MMP-9. The upper (150kDa) band was attributed to dimeric complexes retained under non-reducing conditions. We found no clear differences in the appearance or the density of the bands for any of the MMPs between never-smokers, smokers or atopic smokers. We did not find any differences between BAL1 and BAL2 in any of the groups in this respect either (Table 4 in Supplement 1).

Quantity of gelatinases and gelatinase inhibitors

To confirm the presence of pro-MMP-2 and pro-MMP-9, we quantified MMP-2 and MMP-9 in all BAL samples using ELISA detecting the pro- plus the active forms of each MMP. The ELISA measurements confirmed the zymography results with no pronounced differences seen between BAL1 and BAL2 or between study groups (Fig 2A, B). TIMP-1 and TIMP-2 were analysed with ELISA as well. We found no substantial differences were between BAL1 and BAL2 within or between the study groups for any of the TIMPs (Fig 1C, D). Correlation analysis was performed to make sure that urine cotinine levels, which to some extent differed between the individuals after smoking, did not affect the level of MMPs and TIMPs. None of the concentrations of MMP-9, MMP-2, TIMP-1 or TIMP-2 correlated with cotinine levels.

Net activity of gelatinases

We also determined the net gelatinase activity in BAL fluid, using fluorescent conjugated gelatine that is detectable only upon its degradation. We found no clear difference in net gelatinase activity for BAL1 and BAL2 in atopic or non-atopic smokers (Fig 3). However, in atopic smokers, the net gelatinase activity displayed a higher activity in BAL1 (baseline) samples, compared to never-smokers ($p=0.002$) and non-atopic smokers ($p=0.014$). We also observed a modest increase in gelatinase activity in BAL2 compared with BAL1 in the group of never-smokers ($p=0.022$) (Fig 3).

Gelatinase/gelatinase inhibitor ratios

When ELISA results were used to calculate ratios of MMP-9/ TIMP-1, MMP-2/TIMP-2 and MMP-9/TIMP-2 and MMP-2/TIMP-1, we were unable to reveal any major differences within or between the study groups (Table 5 in Supplement 1).

DISCUSSION

In this study, we assessed effects of acute exposure to tobacco smoke on the identity, quantity and the net activity of local gelatinases, as well as the gelatinase/gelatinase inhibitor ratios in bronchoalveolar lavage fluid from both atopic and non-atopic occasional smokers. The results consistently indicated that under the conditions given in this study acute exposure to tobacco smoke does not cause any pronounced and lasting impact on the local gelatinases or gelatinase inhibitors in the peripheral airways of humans. In addition, the results did not reveal any corresponding impact on the local number of macrophages or neutrophils. These results were found regardless of atopy status. Moreover, our study forwards observations of potential methodological importance as the results indicate that the broncoalveolar lavage procedure itself can exert a certain impact on the accumulation of eosinophils and on the net gelatinase activity locally.

The results in our current study, on both gelatinases and neutrophil counts, correspond well with findings reported in a study on induced sputum from intermittent smokers, in spite of the fact that our current study reflects the peripheral and not the proximal airways (6). The preceding study on proximal airways indicated no substantial changes in sputum MMP-9, TIMP-1 or neutrophil counts 24h after exposure tobacco smoke. However, in contrast to the

current study, the preceding study on the proximal airways did not address MMP-2 and TIMP-2.

The outcome of our study does not support the results of previous studies in mouse models, in which it has been demonstrated that acute exposure to cigarette smoke increases MMP-9 levels and neutrophil counts in bronchoalveolar lavage fluid (13, 14). One reason for this discrepancy may of course be species differences, another reason may be the fact that excessively high doses of tobacco smoke have been administered in the referred studies on mouse models. In our study, we chose what we consider a moderate dose of tobacco smoke (i.e.10 cigarettes during a 48 h period). This dose of tobacco smoke seemed clinically relevant and ethically defendable, considering that the occasional smokers who participated in this study reported that they habitually did not smoke more than 20 cigarettes over a 48h period, a dose we did not want to exceed in our study. Because of this moderate dose of tobacco smoke, one could of course argue that the negative results in this study were due to an insufficient dose of tobacco smoke. Against this argument stands the clear and reproducible increase in the nicotine metabolite cotinine in urine, that we documented for all the included subjects. Apart from being important for the inclusion of study subjects, this documentation of cotinine in urine proves that the utilised dose of tobacco smoke was indeed sufficient to enable systemic dissemination of one key component of tobacco smoke, nicotine. As judged from oral information from the investigated occasional smokers (data not shown), 10-20 hours passed after the last cigarette smoked until the harvest of BAL fluid during the second bronchoscopy. Therefore, we cannot rule out a transient peak in gelatinase mobilisation within this time frame, based upon the results of our current study.

According to previous studies on local quantity and/or activity of MMP-9 and MMP-2 in human airways, gelatinases are increased in COPD and emphysema patients compared to non-smokers (4, 15) and chronic smokers without COPD (3, 16), while the latter do not display significantly different MMP-9 protein levels in relation to never-smokers (17). Notably, a very recent study on smokers with and without COPD reveals that immunoreactivity for MMP-2 protein in peripheral lung tissue correlates with disease severity in patients with COPD rather than to cigarette smoking per se, which is fully in line with our current findings (18). Moreover, COPD patients display higher MMP-9 protein levels in the airways during exacerbations than during the stable phase of their disease (5) and ex-smokers with COPD colonized with bacteria display higher MMP-9 protein levels in the peripheral airways than non-colonized ex-smokers with COPD (19). These observations, together with the observations in our current study, are all compatible with the idea that there is no pronounced impact on gelatinase mobilisation that occurs immediately after tobacco exposure in naïve airways and that such an impact requires repeated exposure to tobacco smoke over time as the pulmonary disease progresses, possibly also affected by co-factors such as infectious agents.

It has previously been demonstrated that MMP-9 protein levels and activity are increased in the proximal airways of patients with asthma and that MMP-9 levels are further enhanced after allergen challenge (11, 20). This previously reported, atopy-related increase in MMP-9 is likely to explain the increase in net gelatinase activity that we observed in the atopic occasional smokers at baseline (prior to smoke exposure) in our study.

Our current study revealed no increased susceptibility to an acute exposure of tobacco smoke in terms of alterations in gelatinases and their inhibitors or the occurrence of inflammatory cells in the peripheral airways of atopic subjects. To the best of our knowledge, our current

study is the first to report on this matter, as most previous studies addressing atopy versus smoking have focused on the tobacco-induced, increased risk of developing atopy and asthma (21, 22) and on the increased severity, inflammation and progress of atopic diseases such as asthma in smokers (23-25).

Interestingly, our current study generated evidence that the BAL procedure *per se* can influence both cell counts and proteins in the lower airways, even though these iatrogenic effects seem modest. In analogy to what has been reported in a previous study on induced sputum (7), we observed that the percentage of eosinophils was increased in never-smokers and in atopic occasional smokers in the second bronchoalveolar lavage sample (BAL2), harvested 14 days after the first bronchoalveolar lavage procedure (BAL1). Moreover, we found a small increase in net gelatinase activity in the second bronchoalveolar lavage sample harvested from never-smokers. These observations encourage extra caution to be taken when designing studies involving repeated bronchoalveolar lavage procedures, and emphasize the need of including a longitudinal control for the procedure *per se*, in addition to the use of baseline controls.

In summary, this study specifically assessed the impact of an acute and moderate dose of tobacco smoke on the concentrations of the quantitatively dominant gelatinases MMP-2 and -9 and their inhibitors TIMP-1 and -2, the gelatinase/gelatinase inhibitor ratio and the net gelatinase activity in bronchoalveolar lavage fluid from occasional smokers. Even though modest, transient alterations cannot be excluded, the main conclusion of our study is that an acute exposure to a moderate dose of tobacco smoke is not sufficient to cause any substantial, lasting impact on gelatinases or their inhibitors in the peripheral airways of humans. Naturally, the fact that we investigated a limited number of subjects means that the risk of

type II errors can not be totally disregarded. However, the three different means of assessing gelatinases in our study all generated results indicating a lack of substantial alterations in gelatinases after one acute exposure to smoke. Moreover, atopy seems to be of no major importance in this specific context. Indeed, our findings are compatible with the fact that it takes many years of tobacco smoking to establish COPD but, importantly, they should not be taken as evidence that occasional exposure to tobacco smoke is harmless. Rather, in view of the results of previous studies on gelatinases in patients with COPD, our current results provide a rationale for hypothesizing that repeated exposure to tobacco smoke is required for the mobilisation of gelatinases in naïve airways and for investigating proteases other than gelatinases as well in this context.

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FIGURE LEGENDS

Figure 1.

Qualitative analysis of identity of dominating gelatinases in human BAL fluid. A representative image of a zymography gel with bands of 70, 90 and 150kDa corresponding to pro-MMP2 and pro-MMP9 and (presumably) dimeric complexes retained under non-reducing conditions. All samples are from the second BAL procedure in each subject (NS= never smoker not exposed to tobacco smoke, S= occasional smoker exposed to tobacco smoke, AS= atopic occasional smoker exposed to tobacco smoke)

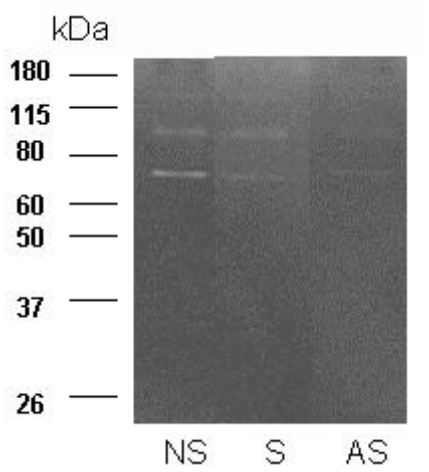
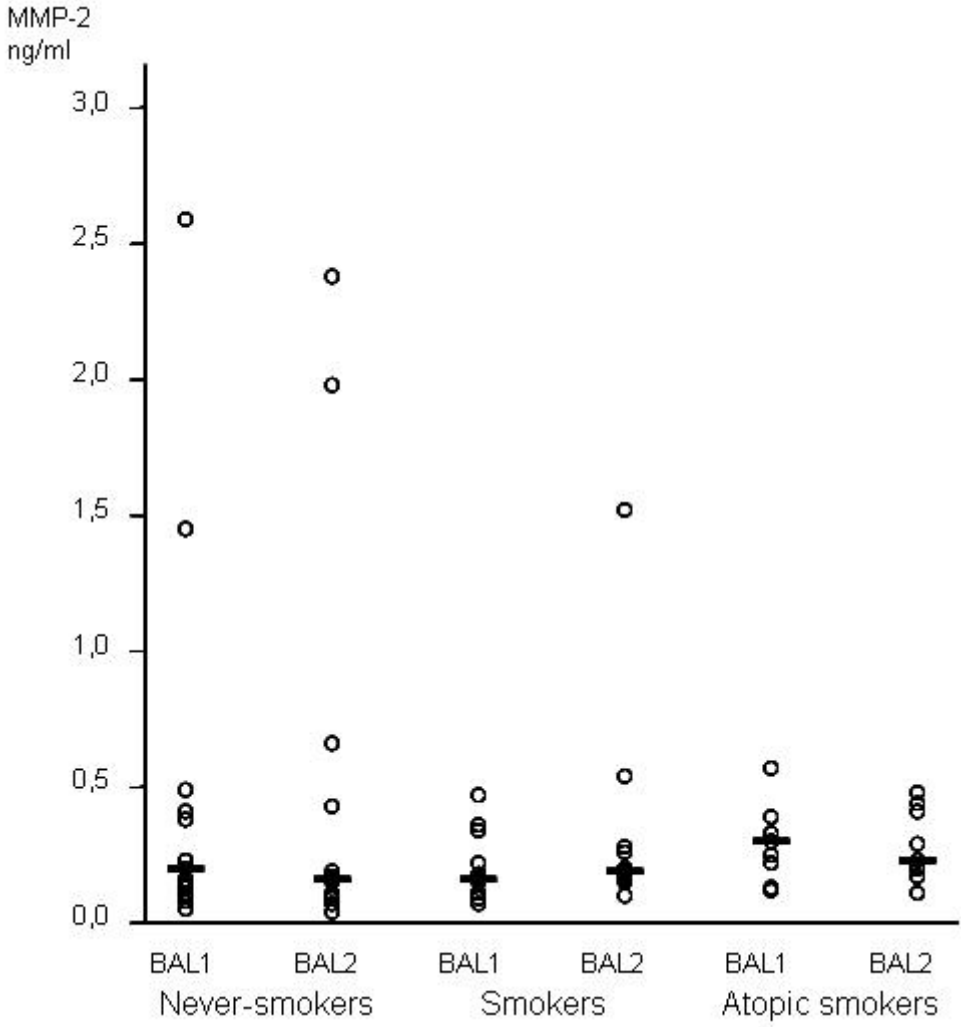
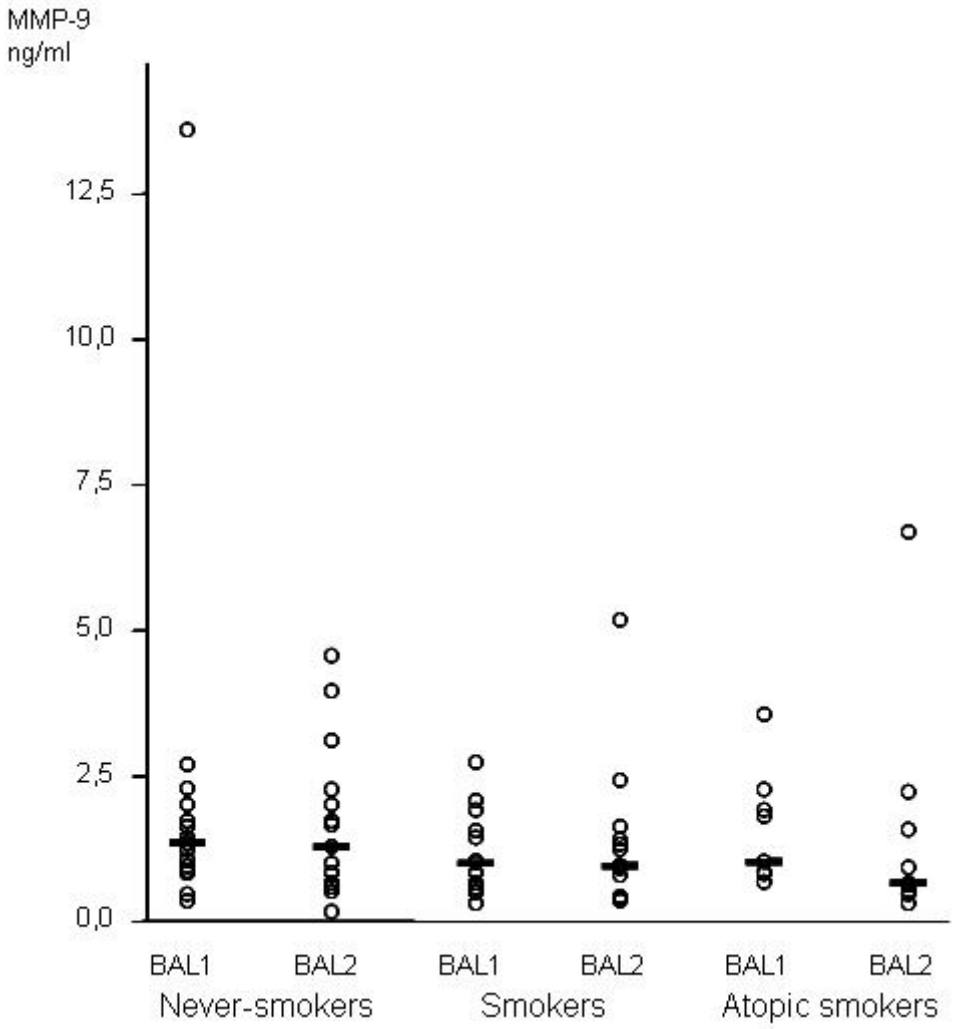
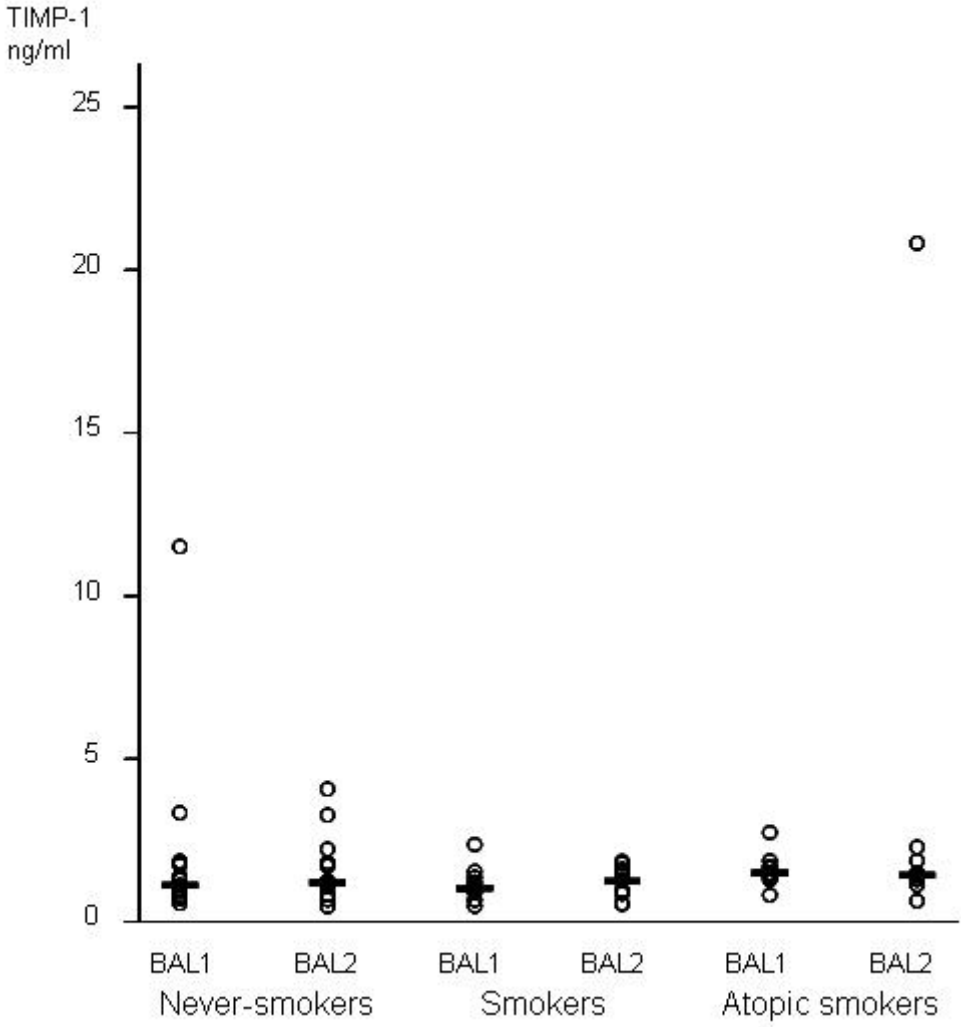


Figure 2.

Quantitative analysis of gelatinases and gelatinase inhibitors in human BAL fluid. ELISA was utilised to measure concentrations of total MMP-2 (A), MMP-9 (B), TIMP-1 (C) and TIMP-2 (D) in BAL samples before (BAL1) and after (BAL2) smoking in non-atopic occasional smokers (n=13) and atopic occasional smokers (n=9) and in corresponding BAL samples from a control group of never-smokers (n=15) not exposed to tobacco smoke between bronchoscopies. No significant differences were found between study groups or before and after smoking.


MMP-2
ng/ml
3,0
2,5
2,0
1,5
1,0
0,5
0,0
BAL1
BAL2
BAL1
BAL2
BAL1
BAL2
Never-smokers
Smokers
Atopic smokers


MMP-9
ng/ml
12,5
10,0
7,5
5,0
2,5
0,0
BAL1
BAL2
BAL1
BAL2
BAL1
BAL2
Never-smokers
Smokers
Atopic smokers


TIMP-1
ng/ml
25
20
15
10
5
0
BAL1
BAL2
BAL1
BAL2
BAL1
BAL2
Never-smokers
Smokers
Atopic smokers

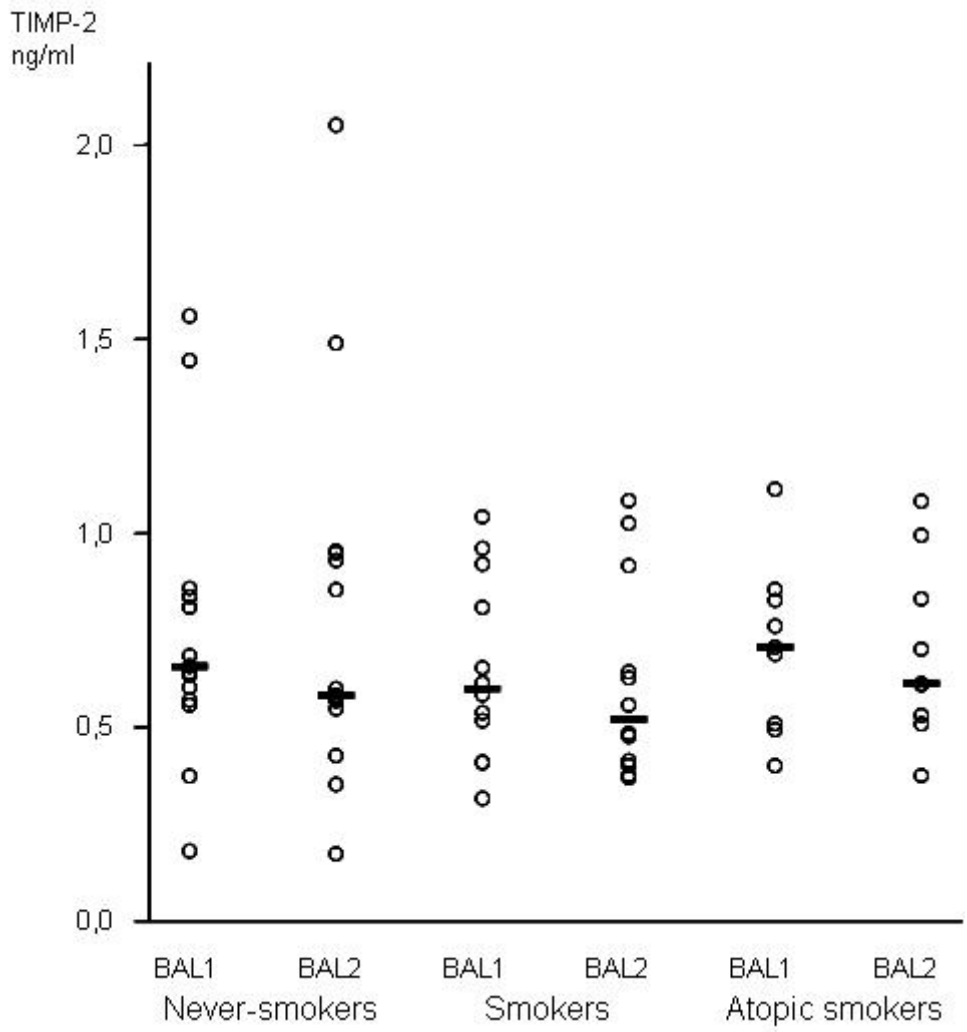
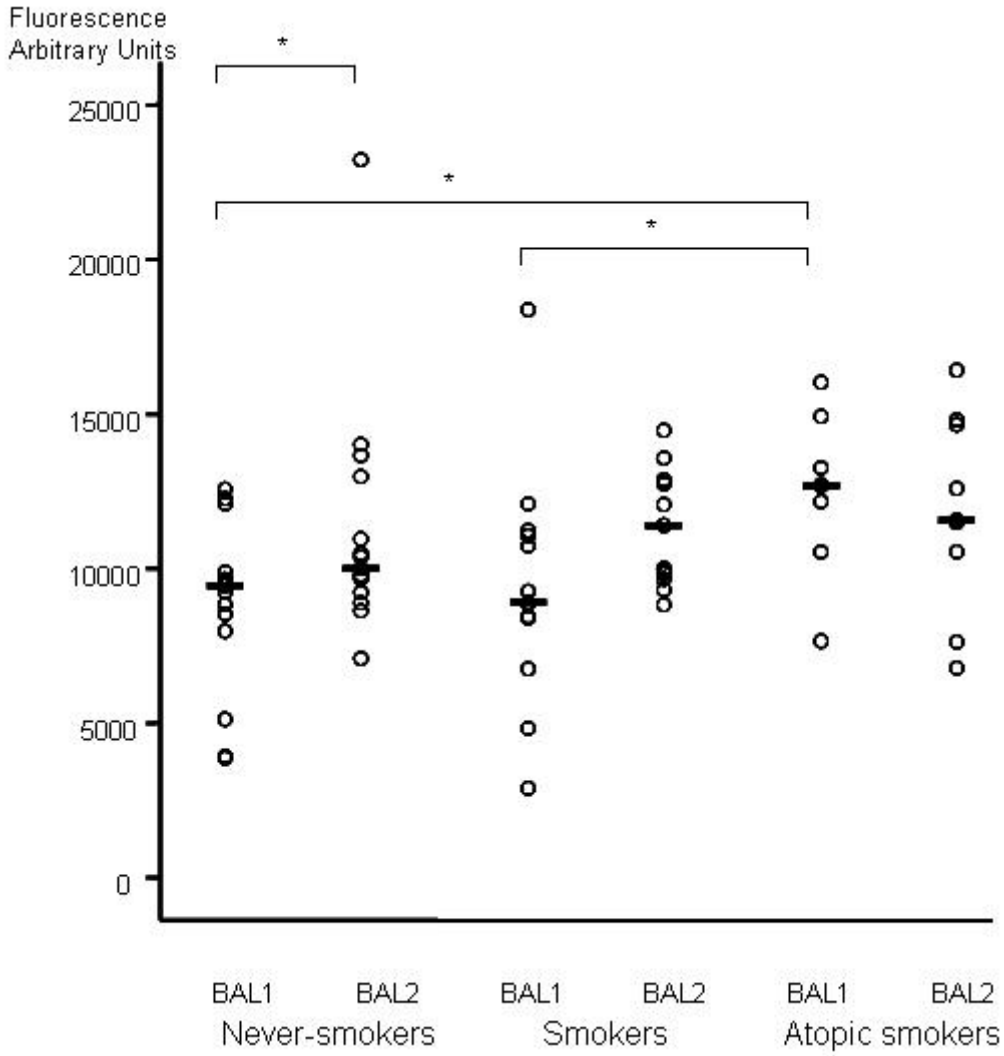


Figure 3.

Net gelatinase activity in human BAL samples. Samples were harvested before (BAL1) and after (BAL2) controlled smoking in non-atopic occasional smokers (n=13) and atopic occasional smokers (n=9) and in a control group of never-smokers (n=15). Baseline levels (BAL1) of net gelatinase activity was higher in atopic than in non-atopic occasional smokers (p=0.014) and never-smokers (p=0.002). In the control group of never-smokers alone, this net gelatinase activity was higher in BAL2 than in BAL1 (p=0.022).


Fluorescence
Arbitrary Units
25000
20000
15000
10000
5000
0
*
*
*
BAL1
BAL2
BAL1
BAL2
BAL1
BAL2
Never-smokers
Smokers
Atopic smokers

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