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Identification of MMP-9 as a biomarker for detecting progression of chronic obstructive pulmonary disease.

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Running Title: MMP-9 as a COPD progression marker.

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a complex immunological disease with multiple pathological features that is primarily induced by smoking together with additional genetic risk factors. COPD is frequently underdiagnosed; forced expiratory volume in the first second (FEV1) is considered to be the main diagnostic measure for COPD, yet it is insufficiently sensitive to monitor disease progression. Biomarkers capable of monitoring COPD progression and severity are needed. In this report, we evaluated matrix metalloproteinase-9 (MMP-9) as an early marker for the detection and staging of COPD, by assessing the mRNA levels of MMP-9 in peripheral blood samples collected from 22 COPD patients, 6 asymptomatic smokers and 5 healthy controls. Our results demonstrate that the mRNA levels of MMP-9 increased more than two-folds in severe COPD relative to non-COPD smokers or moderate COPD groups. Moreover, in the very severe COPD group, MMP-9 mRNA levels showed a 4-fold increase relative to the non-COPD smokers or the moderate COPD groups, while there was a mild increase (~ 40%) when compared to the severe COPD group. Taken together, our results suggest that MMP-9 serves as a biomarker for the grade and severity of COPD.

Keywords:

Biomarker, Chronic obstructive pulmonary disease, Matrix Metalloproteinase-9, mRNA expression, quantitative PCR, SYBR green.

Introduction

COPD is a collective term describing two separate chronic lung diseases that adversely affect airflow: chronic bronchitis and emphysema (Ning *et al.* 2004). COPD typically develops over the course of many years (Cosio *et al.* 2009; Bruce and McEvoy 2007). COPD is presently the fourth leading cause of death worldwide, but the world health organization (WHO) predicts that it will become the third leading cause of death by 2030 (WHO, 2008). Many people suffer from this disease for years, and die prematurely from it or from its complications (Decramer *et al.* 2012; GOLD 2011). Prevalence surveys suggest that almost one quarter of adults aged 40 years and older have mild airflow obstruction (Mannino and Buist 2007; Buist *et al.* 2007; and Menezes *et al.* 2005).

COPD risk factors are actively studied. Smoking is the major risk factor and the predominant cause of COPD, and is implicated in 90 % of COPD cases (Bruce and McEvoy 2007; and Doherty 2002). However, only 10–20 % of the smokers develop COPD, pointing to one or more additional risk factor, such as genetic susceptibility. Several genes have been identified as modifiers of individual susceptibility to COPD, such as the genes encoding proteases (e.g. Matrix Metalloproteinases (MMPs)), anti-proteases (e.g. alpha-1-antitrypsin (A1A)), inflammatory mediators (e.g. interleukin-2), proteins with antioxidant properties (e.g. extracellular superoxide dismutase (SOD)), enzymes that metabolize xenobiotic compounds (e.g. Glutathione-S-transferases (GSTs)) and proinflammatory mediator such as tumor necrosis factor- α (TNF- α) (Molfinio and Jeffery 2006; Hirvonen 2009; Harrison *et al.*, 1997; Keatings *et al.* 2000; Sandford *et al.* 2001; Kucukaycan *et al.* 2002; Celedon *et al.* 2004; Young *et al.* 2006; and Stockley 2014). However, additional factors also contribute, including exposure to both indoor and outdoor air pollutants and early life exposure to both infectious and non-infectious agents represent risk factors that might play an important role in the development and advancement of COPD (Bruce and McEvoy 2007; and Doherty 2002).

COPD is frequently underdiagnosed due to the lack of definitive diagnostic biomarkers. The major marker utilized to monitor COPD patients is “forced expiratory volume in the first second (FEV1)” (D'Armiento *et al.* 2009). FEV1 is used to diagnose the stage of COPD and to predict COPD mortality (Decramer *et al.* 2005). Taking into consideration that COPD onset is relatively asymptomatic, patients that are at risk or in the early stages of the disease are not usually monitored by FEV1 measurements. As a result, COPD is typically diagnosed only after disease progression (Rabe *et al.* 2007). Consequently, there is a considerable need for new reliable and reproducible markers specific to COPD that are able to diagnose disease severity and foretell progression (D'Armiento *et al.* 2009).

Matrix MetalloProteinases are prominent proteolytic molecules released by neutrophils and alveolar macrophages during the inflammatory events in COPD. MMP-9 which is the predominant protease in alveolar tissues, has received considerable attention among many MMPs, due to its ease of detection and quantification, suggesting it could be used as a COPD biomarker (Kumar *et al.* 2011). MMP-9 is a zinc dependent endopeptidase that is involved in an array of normal and pathological processes including remodeling of extracellular matrix (ECM), development, inflammatory response, and carcinoma progression (Muroski *et al.* 2008). MMP-9 is synthesized and secreted in monomeric form as a zymogen that is subsequently activated via a protease cascade (Cuzner and Opdenakker 1999). MMP-9 is not produced in healthy lung. However, during inflammatory events of COPD, bronchial epithelial cells, clara cells, alveolar type II cells, fibroblasts, smooth muscle cells, and endothelial cells produce MMP-9 as well as leukocytes in the lung (Greenlee *et al.* 2007).

Evidence supporting the role of MMP-9 in COPD includes the enzyme's *in vitro* degradation of elastin and Alpha-1-Antitrypsin, two proteins involved in the structural integrity and preservation of pulmonary tissues (Muroski *et al.* 2008). Within COPD, MMP-9 degrades elastin and facilitates further lung damage, as it is secreted by alveolar macrophages

and neutrophils that act as facilitators of the inflammatory response. MMP-9 also inactivates Alpha-1-Antitrypsin, which typically serves a protective function by inhibiting Neutrophil Elastase, as it is also able to cleave elastin, a crucial structural component of the lungs. Taken together, these mechanisms support roles for MMP-9 as a critical mediator in COPD.

The current study aims to evaluate MMP-9 as biomarker for COPD early detection and staging by comparing its relative mRNA expression levels isolated from peripheral blood white blood cells of normal, asymptomatic smokers, moderate COPD, severe COPD and very severe COPD patients. The data presented in this report support the use of MMP-9 as a biomarker to differentiate between moderate, severe and very severe COPD. However, our results suggest that MMP-9 mRNA levels cannot be used for early detection of the disease.

Subjects and Methods

I. subjects

33 subjects were involved in this study classified into 3 major groups. Group (1), healthy non-smoking controls (age 41 to 72 years; n=5); group (2), non-COPD Smokers (age 40 to 63; n=6); and group (3); COPD patients (age 42 to 76 years; n=22) enrolled at the Chest Department and Clinic at Ain Shams University Hospitals.

COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (GOLD 2011). The COPD patients were diagnosed by clinical signs, symptoms, local chest examination and the diagnosis was confirmed by spirometric examination and a FEV1/FVC with a ratio of < 70%). COPD patients were further classified based on the post-bronchodilator FEV1% predicted values into 3 subgroups. Group 3A represented the moderate COPD group (n=8), where the FEV1% was between 50% and 80%; group 3B represented the severe COPD group (n=7), where the FEV1% was between 30% and 50%; while group 3C represented the very severe COPD group (n=7), where the FEV1%

was less than 30%. The selected COPD subjects were not diagnosed with any coexisting pulmonary diseases, like; bronchiectasis, tuberculosis, asthma, or lung cancer.

The study was approved by the local research ethics committee and was performed in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards, and all subjects signed written informed consent.

II. Methods

Lung function testing

The FEV1, forced vital capacity (FVC), and FEV1/FVC ratio were determined using a Jaeger spirometer and were performed at the Chest Department at Ain Shams University Hospitals. Values are expressed as percentages of the predicted normal values for age, sex, and height according to the European Community for Steel and Coal guidelines (Quanjer 1983; Sherril *et al.* 1992).

White blood cells isolation, total RNA preparation and cDNA synthesis

White blood cells were isolated from blood according to McCoy 1998. Blood samples (4 ml) were collected using heparin vacutainer tubes followed by addition of 10 ml red blood cells lysis buffer (10 mM KHCO₃, 150 mM NH₄Cl, 1 mM EDTA). The mixture was incubated for 10 min at RT, followed by centrifugation for 5 min at 2,000 rpm. Following centrifugation, the cleared supernatant was discarded and the cells were washed twice using 1 ml PBS followed by centrifugation for 5 min at 2,000 rpm (McCoy 1998). The recovered white blood cells pellet were ruptured in 1 ml Biozol® reagent (BioRad Cat.# 161-0100), and total RNA was extracted according to the manufacturer's recommendations.

Reverse transcription of the extracted RNA (1 µg) was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific* Fermentas Cat # K1612) according to the manufacturer's recommendations.

Primers

Primers (Table 1) used in this study to amplify matrix metalloproteinase-9 (MMP-9) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using BLAST tool.

Conventional PCR detection of MMP-9 and GAPDH mRNA

The conventional PCR for MMP-9 and GAPDH was performed in 25 µl reaction using GoTaq® Flexi DNA Polymerase (Promega). The reaction mix contained 5 µl of 5X green GoTaq® Flexi Buffer, 1.5 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs, 0.4 µl of the corresponding forward primer (20 µM), 0.4 µl of the corresponding reverse primer (20 µM), 0.75 unit of the polymerase, 1 µl of template cDNA and 16 µl nuclease free water. PCR performed for 35 cycles with initial denaturation step at 94°C for 5 min. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, elongation at 72°C for 30 sec followed by 10 min of terminal extension at 72°C after the completion of the last cycle. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed under ultraviolet light.

Real time PCR detection of MMP-9 and GAPDH gene expressions

Real time PCR was performed using a Mx3005P quantitative PCR system (Agilent Technologies) with Brilliant II SYBR® Green QPCR Master Mix (Agilent Cat # 600828). The reaction mix (25 µl) contained 12.5 µl of 2X Brilliant II SYBR Green QPCR master mix, 0.4 µl forward primer (20 µM), 0.4 µl of reverse primer (20 µM), 1 µl of 1:10 diluted cDNA template and 10.7 µl nuclease free water. The PCR protocol consisted of 40 amplification cycles with initial denaturation at 95°C for 10 min. Each cycle consists of denaturation at

95°C for 30 sec, annealing at 63°C for 30 sec, and extension at 72°C for 30 sec. Dissociation curves generated by incubating the products for 1 min at 95°C, followed by 30 sec at 55°C and finally 30 sec at 95°C. Data were analyzed using the MXpro QPCR software (Agilent Technologies) to estimate cycle threshold (C_T) values and dissociation curves to estimate the optimal melting temperatures for all reactions. In all experiments, we included two additional negative controls: 1) RT-control, which consisted of a PCR reaction lacking reverse transcriptase, and 2) a no template control (NTC), which used water in place of the cDNA template.

Statistical analysis

Fold change in gene expression (FGE) of MMP-9 was calculated according to Livak and Schmittgen 2001. Briefly, the ΔC_T value for each group was calculated by subtracting the C_T of GAPDH from the C_T of MMP-9. The $\Delta\Delta C_T$ was calculated by subtracting the mean ΔC_T of the healthy non-smoking control group from the mean ΔC_T of each of the studied group. FGE was calculated by applying the following formula: $FGE = (2^{-\Delta\Delta C_T})$ (Livak and Schmittgen 2001). The level of gene expression was considered down-regulated if the FGE value was lower than 1.0, up-regulated if the FGE value was greater than 1.0, and unchanged if the FGE was equal to 1.0. The data were statistically analyzed using SPSS15.0 software (Statistical Software Package for Windows). Values were expressed as mean $\pm$ standard deviation (SD). Differences in the numerical data between every two groups were evaluated using Mann-Whitney U test, and between the five groups using Kruskal Wallis test. The significance among the groups was compared at the level of significance $P=0.05$ as follows: $P<0.05$ = significant, $P< 0.01$ or 0.001 = highly significant, and $P > 0.05$ = non-significant (NS).

Results

Conventional PCR analysis

Conventional PCR was carried out on the 33 cDNA samples followed by agarose electrophoresis prior to qPCR to check the quality of the cDNA samples, and to assess the specificity of the primer pairs designed for the 2 genes under study, GAPDH and MMP-9. The data presented in figures 1 illustrates results of agarose gel electrophoresis for PCR amplifications of GAPDH (amplicon size=150 bp) and MMP-9 (amplicon size=136 bp) using the specific primers noted in table 1. The results suggested that the primer sets for GAPDH and MMP-9 were specific, and that the cDNA preparation did not have any genomic DNA contamination.

MMP-9 expression measurements

Gene amplification curves from RT-PCR of MMP-9 and GAPDH cDNA shown in figure 2, panels A, C, E, G and I represents the plot of Delta Rn (ΔR_n) versus the cycle number. The dissociation curve for each gene relative to the melting temperature (T_m) was determined (Figure 2, panels B, D, F, H and J). The C_T values of the GAPDH and MMP-9 presented in table 2 and figure 3. Levels of MMP-9 Fold of gene expression were calculated for the non-COPD smoking group, moderate COPD group, the severe COPD group, and the very severe COPD group as described in Material and Methods.

The data presented in table 3 and figure 4 showed variations in the mean FEG values between the three COPD grades (moderate, severe and very severe) with a trend suggesting the greater the decline in FEV_1 , the greater the MMP-9 expression. No significant variation in MMP-9 expression levels was observed between the non-COPD smokers (0.44 ± 0.2) and the moderate COPD groups (0.39 ± 0.15). In both of these groups, MMP-9 levels were similarly down-regulated relative to the very severe COPD group. The MMP-9 level of expression in severe COPD group (1.13 ± 0.12) increased more than two- fold compared to the non-COPD

smokers and the moderate COPD groups. In addition, the MMP-9 level of expression in the very severe COPD group (1.66 ± 0.55) increased more than four-fold compared to the non-COPD smokers and the moderate COPD groups. Finally, there was an ~40% increase in MMP-9 levels observed in the moderate COPD group compared to the severe COPD group.

Differences in MMP-9 FGE were compared between every two studied groups using Mann-Whitney U test and between the five tested groups using Kruskal Wallis test, and significant differences are summarized in table 3. A highly significant increase of MMP-9 FGE between the non-COPD smokers *vs* the severe COPD group ($P = 0.003 < 0.01$), the non-COPD smokers *vs* the very severe COPD group ($P = 0.003 < 0.01$), the moderate COPD group *vs* the severe COPD group ($P = 0.001 < 0.01$), the moderate COPD group *vs* the very severe COPD group ($P = 0.001 < 0.01$); and the severe COPD group *vs* the very severe COPD group ($P = 0.021 < 0.05$) was observed, while the Kruskal Wallis test showed a significant change in MMP-9 FGE among the four studied groups.

Discussion

COPD is a condition primarily caused by the inhalation of noxious particles or gases (usually cigarette smoke) leading to inflammation and remodeling in the large and small airways, and destruction of the lung parenchyma leading to emphysema. COPD diagnosis and severity are assessed by spirometric measurements (FEV1, FEV1/FVC ratio). However, there is a considerable evidence supporting the view that COPD is under-diagnosis, especially in the mild and moderate groups, and that FEV1 is an insufficiently sensitive biomarker to accurately diagnose the very early onset of disease (Nicholas 2013). In addition to spirometric diagnosis, several previous studies have explored the relationships between different biomarkers and reduced lung function or COPD. However, the results have varied significantly between studies because of differences between laboratory methods.

MMP-9 (Gelatinase B, 92-kD type IV collagenase) is an MMP that is present in low quantities in the healthy adult lung, but much more abundant in several lung diseases, including asthma, idiopathic pulmonary fibrosis (IPF), and COPD (Atkinson and Senior 2003). MMP-9 is secreted along with its inhibitor TIMP-1 which can bind to both the inactive and active form of MMP-9 (Ram *et al.* 2004). Although many MMPs have been proposed to play a role in lung pathology, MMP-9 has received a considerable attention concerning its relationship to COPD identification, diagnosis, pathogenesis, and disease management (Engstrom *et al.* 2012; Dickens *et al.* 2011; Kumar *et al.* 2011; and Koczulla *et al.* 2012). Compared to healthy controls, COPD patients display increased protein levels of MMP-9 were reported in sputum, plasma and serum (Brajer *et al.* 2008; Kang *et al.* 2003). Monocytes from COPD patients also have increased activity as these releases more MMP-9 but less IL-8 than control subjects (Aldonyte *et al.* 2003). The MMP-9/TIMP-1 ratio positively correlates with FEV1 in COPD patients, who have low FEV1, have lower ratios than control subjects (Higashimoto *et al.* 2005 and Vignola *et al.* 1998).

In the current study, we determined that MMP-9 expression levels varied significantly between moderate, severe, and very severe COPD patients, relative to healthy non-smoking controls. However, up-regulation of MMP-9 observed only in severe and very severe COPD groups. Consistent with the data presented in this study; an elevation in MMP-9 protein levels in induced sputum of symptomatic smokers with normal FEV1/FVC (Stage 0 COPD, GOLD criteria), as well as asymptomatic smokers, and healthy non-smoking controls by ELISA were reported in stage 0 COPD group and asymptomatic smokers than healthy non-smoking subjects (Ilumets *et al.* 2007). Furthermore, in a study conducted by Pinto-Plata *et al.* 2007, the authors compared the serum proteomic profiles for 143 serum biomarkers in patients with severe COPD with those from age and sex-matched controls using a novel protein microarray platform (PMP) technology, and found 43 biomarkers, including TIMP-1 and MMP-9, were

different between patients and controls. Amongst those examined, serum MMP-9 showed the strongest correlation with exacerbation rates, suggesting that MMP-9 may be related to the rapid decline of FEV1 with regard to the rate of exacerbation (Pinto-Plata *et al.* 2007). Studies of MMP-9 gene expression in COPD patients, to some extent, supported the findings reported in proteomics studies, where an up-regulation of MMP-9 gene expression found in the lung parenchymal cells and correlated with the progression and severity of COPD (Gosselink *et al.* 2010).

Llinàs *et al.* 2011, compared the mRNA expression level of genes involved in inflammation, tissue remodeling, and vessel maintenance between COPD patients with moderate, severe and very severe grades and non-COPD smokers. The authors used mRNA isolated from lung tissue. Based on their results, they reported; an up-regulation in MMP-9 expression in severe to very severe COPD grades as compared to moderate COPD and non-COPD smokers. In contrast, MMP-9 expression did not differ between moderate COPD and non-COPD smokers. These findings are consistent with our observations; since we also noticed a significant increase in MMP-9 expression in the severe COPD group, which was further enhanced in the very severe COPD group. In contrast, we failed to observe a significant difference in MMP-9 expression between moderate COPD patients and non-COPD smokers (Llinas *et al.* 2011). On the other hand, while MMP-9 over-expression was the general response observed in this study, MMP-9 down-regulation was also observed in non-COPD smokers and moderate COPD patients. A similar observation reported by Churg *et al.* 2012, while a drop in MMP-9 expression observed in mice chronically exposed to cigarette smoke condensate as compared to air-exposed mice (Churg *et al.* 2012). However, different results were reported with MMP-9 knockout mice. These mice showed a similar inflammatory response profile and developed the same severity of cigarette smoke induced emphysema as did strain-matched wild-type mice. This study demonstrated that MMP-9

mRNA levels did not correlate with markers of continuing lung damage or with local degrees of emphysema (Atkinson *et al.* 2011).

To explain the down-regulation of MMP-9 expression levels observed in the current study, one can speculate that the defense mechanisms fostered by the human body to face the primary smoking-induced elevation of MMP-9 expression may be strong enough to cause temporary down-regulation of MMP-9, as observed in moderate COPD and asymptomatic smoking individuals. Meanwhile, the increase in production of interferon-gamma (INF- γ), as one of the responses to smoke exposure, causes a reduction in MMP-9 expression (Sanceau *et al.* 2002; Spira *et al.* 2004; Golpon *et al.* 2004). However, continuous exposure of lungs to noxious particles in cigarette smoke accompanied by continuous release of free radicals and accelerated inflammatory responses would cause deterioration in the body defense mechanisms in favor of increasing MMP-9 expression, which was observed in the severe and the very severe COPD grades. In conclusion, this work represents a reproducible, convenient and simple method for use of MMP-9 as a biomarker for the COPD progression. MMP-9 can be relied upon to determine the grade and severity of COPD. However, the validity of this marker for early diagnosis of the disease is questionable. The same approach can be used to identify other biomarker that may be used for early and reliable detection of COPD.

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Competing Interests

Authors have declared that no competing interest exists.

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Table 1: Primer sequences used for PCR detection of MMP-9 and GAPDH gene expression.

Gene	Primer sequence
GAPDH-Forward	5'-GAAGGTCGGAGTCAACGGATTT-3'
GAPDH-Reverse	5'-CATGGGTGGAATCATATTGGAAC-3'
MMP-9- Forward	5'-CGGCGCCTCTGGAGGTTCG-3'
MMP-9-Reverse	5'-GCAGAAATAGGCTTTCTCTCGG-3'

Table 2: The C_T values for GAPDH and MMP-9 within the studied groups.

Group Samples	Healthy Non Smoking Control		Non-COPD Smokers		Moderate COPD		Severe COPD		Very Severe COPD	
	GAPDH	MMP-9	GAPDH	MMP-9	GAPDH	MMP-9	GAPDH	MMP-9	GAPDH	MMP-9
1	24.09	26.58	21.73	26.79	21.54	26.19	22.63	24.85	22.43	24.56
2	24.72	26.64	20.3	23.93	19.45	23.98	21.46	24	19.55	21.99
3	21.12	24.56	21.22	24.78	22.21	25.87	18.55	21.02	18.54	20.85
4	24.27	26.24	19.89	24.18	21.25	24.98	18.32	20.59	21.62	23.29
5	20.35	23.34	19.47	23.32	23.26	28.03	19.91	22.12	21.41	22.52
6			27.08	24.13	21.37	25.45	18.96	21.4	21.39	23.23
7					21.25	24.85	18.71	21.26	24.15	25.88
8					20.88	24.05				
Average	22.91	25.472	21.61	24.52	21.40	25.42	19.791	22.177	21.298	23.188
St. Dev.	2.01	1.46	2.80	1.20	1.08	1.31	1.65	1.62	1.83	1.65

Table 3: The mean FGE for MMP-9 within the studied groups.

	Non-COPD Smokers	Moderate COPD	Severe COPD	Very Severe COPD
Mean of FGE	0.44 ± 0.2	0.39 ± 0.15	1.13 ± 0.12	1.66 ± 0.55

Table 4: Comparison of MMP-9 FGE between all studied groups using Mann-Whitney U test and Kruskal Wallis tests.

	Mann-Whitney U test			Kruskal Wallis test
Groups	Moderate COPD	Severe COPD	Very Severe COPD	
Non-COPD Smokers	NS	P= 0.003**	P= 0.003**	P = 0.0001**
Moderate COPD		P= 0.001**	P= 0.001**	
Severe COPD			P= 0.021*	
Very Severe COPD				
(*) = Significant. (**) = High significant. (NS) = Non Significant.				

Figure Legends

Figure 1: Agarose gel electrophoresis for GAPDH and MMP-9 PCR amplification.

PCR products were separated on 2% agarose gel; and were visualized by ethidium bromide staining. Lane (1) is a DNA ladder, lane (2) is the positive control, lane (3) is the no template control (NTC), lane (4) is the -RT control. Lanes 5 to 15 are randomly selected samples from each of the studied groups, including COPD groups, non-COPD smokers, and healthy non-smoking control group.

Figure 2: MMP-9 amplification plots as well as their corresponding dissociation curves for all studied groups. Graphs showing delta Rn versus cycle number for the amplification plots (panels A, C, E, G, I) and the first derivatives of reference dye-normalized fluorescence reading multiplied by -1 versus the temperature for the dissociation curves (panels B, D, F, H, J) for representative samples studied for the MMP-9 and GAPDH primer sets. The brown horizontal line in panels A, C, E, G and I indicates the cycle threshold value.

Figure 3: C_T Values of the MMP-9 and GAPDH within the studied groups. Graph showing the average C_T values for MMP-9 and GAPDH (represented in table 2) in the healthy non smoking controls, non-COPD smokers, moderate COPD, severe COPD and very severe COPD groups. Error bars represent the standard deviation.

Figure 4: MMP-9 expression levels varies within the studied groups. Comparison of the MMP-9 fold of gene expression data (represented in table 3) in the non-COPD smokers, moderate COPD, severe COPD and very severe COPD groups. Error bars represent the standard deviation.

Figure 1
99x33mm (300 x 300 DPI)

Draft

Figure 2
246x303mm (300 x 300 DPI)

Figure 3
170x99mm (300 x 300 DPI)

Figure 4
173x108mm (300 x 300 DPI)