Low serum double-stranded DNA levels are associated with higher survival rates in severe COPD patients

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Shareable abstract (@ERSpublications)
COPD patients with low serum levels of double-stranded DNA (dsDNA) have a better prognosis for survival compared to severe COPD patients with high serum levels of dsDNA. This study demonstrates the potential of using dsDNA as a COPD biomarker. https://bit.ly/491dL7Z

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Abstract

Introduction Damage-associated molecular patterns (DAMPs) are endogenous danger signals that alert and activate the immune system upon cellular damage or death. It has previously been shown that DAMP release is increased in patients with COPD, leading to higher levels in extracellular fluids such as serum. In the current study we investigated whether the serum levels of DAMPs were associated with survival rates in COPD patients.

Methods A panel of seven DAMPs, consisting of HMGB1, fibrinogen, α-defensin, heat shock protein 70, S100A8, galectin-9 and double-stranded DNA (dsDNA), was measured in serum of 949 severe COPD patients. Maximally selected rank statistics was used to define cut-off values and a Cox proportional hazards model was used to evaluate the effect of high or low DAMP levels on 4-year survival. For DAMPs that were found to affect survival significantly, baseline characteristics were compared between the two DAMP groups.

Results Out of the seven DAMPs, only dsDNA was significantly associated with 4-year survival. Patients with elevated serum level of dsDNA had higher 4-year mortality rates, lower FEV_1 % predicted values and higher emphysema scores.

Discussion In conclusion, in a clinical cohort of 949 patients with moderate-to-severe COPD, elevated serum levels of dsDNA were associated with a higher risk of death. This study further illustrates the potential role of circulating DAMPs, such as dsDNA, in the progression of COPD. Together, the results of this study suggest that levels of circulating dsDNA might serve as an additional prognostic biomarker for survival in COPD patients.

Introduction

COPD is a heterogeneous and often progressive respiratory disease characterised by persistent airflow limitation, respiratory symptoms and structural lung changes, such as airways disease (bronchitis), emphysema or both [1]. It can significantly impact quality of life, lead to hospitalisations and ultimately result in death. Currently, COPD is the third leading cause of death worldwide [2]. The disease is caused by a combination of the chronic inhalation of toxic particles and gases and genetic susceptibility [3]. Cigarette smoking is one of the most important risk factors for COPD, although recent reports indicate that in low- and middle-income countries up to 70% of the COPD cases can be caused by other risk factors, such as indoor and outdoor air pollution [4].

The inhalation of toxic gases and particles causes damage to the epithelial layer of the lungs, subsequently inducing the release of pro-inflammatory damage-associated molecular patterns (DAMPs) from these

damaged epithelial cells. DAMPs have been postulated to play an important role in the pathophysiology of COPD, by initiating and maintaining the inflammatory status of the airways [5–8]. The structure of DAMPs resemble that of pathogen-associated molecular patterns and alert and activate the innate immune system in a similar manner as a pathogen would. DAMPs consist of a wide variety of molecules, which have in common that, upon release from a damaged or dead cell, they can activate pattern recognition receptors, such as toll-like receptors or the receptor for advanced glycation end-products (RAGE), to initiate a pro-inflammatory response [7, 9]. Previously it was shown that smoking induces the release of DAMPs [8, 10–15]. Likewise, it was shown that other sources of inhaled toxicants such as diesel exhaust particles and particulate matter also induce the release of DAMPs [16–19]. It is likely that COPD patients are genetically more susceptible to exposure-driven DAMP release, as the release of DAMPs has a strong genetic component [13, 20, 21]. Furthermore, the gene encoding RAGE, one of the most important DAMP receptors, has consistently been found as a susceptibility gene for COPD [22–24]. Consequently, the levels of several DAMPs such as high mobility group box 1 (HMGB1), LL-37, defensins, galectins and S100A8/A9 have been found to be increased locally in bronchoalveolar lavage fluid, as well as systemically in serum or plasma [5, 7, 25–27]. The importance of DAMPs in the pathophysiology of COPD is further demonstrated by the positive effects of inhibition of RAGE on airway inflammation and alveolar tissue damage [28–30].

Although several studies show that the levels of DAMPs in sputum [25], bronchoalveolar lavage fluid [5] and serum [5] associate with the severity of COPD, no information is available on the association between circulating DAMPs in COPD patients and survival. The aim of this study was to investigate whether the levels of DAMPs in serum were associated with survival in COPD patients. To this end, we assessed the serum levels of a panel of DAMPs consisting of HMGB1, α-defensin, heat shock protein 70 (Hsp70), S100A8, galectin-9 and double-stranded DNA (dsDNA), encompassing a wide variety of different DAMPs, in a cohort of 949 severe COPD patients and investigated their association with 4 year survival rates.

Methods

Study population

Data were collected from patients who were enrolled in the Groningen severe COPD cohort (ClinicalTrials.gov identifier: NCT04023409) and from whom additional serum was collected and at least one of the DAMPs of interest was measured. This cohort contained prospectively enrolled patients with COPD who visited the University Medical Center Groningen (UMCG, the Netherlands) for a second opinion consult to assess their eligibility for a lung volume reduction treatment, between August 2014 and August 2019. All patients used in this study were fully characterised, with extensive lung function data, computed tomography scans, blood inflammation parameters and questionnaire data. Vital status was extracted from the Dutch municipal personal records database on 25 July 2023 and 4-year survival status was determined for all included patients. This cohort was approved by the ethics committee of the University Medical Centre Groningen (EC-number: 2014/102), and all patients provided written informed consent.

Damage-associated molecular patterns

The DAMPs of interest that were measured in this study were HMGB1, α-defensin, Hsp70, S100 calcium-binding protein A8 (S100A8), galectin-9, dsDNA and fibrinogen. These DAMPs were measured in patients from whom additional serum was collected using the following assays, all performed according to manufacturer's protocols: HMGB1 (HMGB1 detection kit #6010, Chondrex Inc, Woodinville, WA, USA), α-defensin (Human α-Defensin 1 DuoSet ELISA, DY8198–05, R&D Systems, Minneapolis, MN, USA), Hsp70 (Human HSP70/HSPA1A DuoSet ELISA, DY1663–05, R&D Systems), S100A8 (Human S100A8 DuoSet ELISA, DY4570–05, R&D Systems), galectin-9 (Human Galectin-9 DuoSet ELISA, DY2045, R&D Systems) and dsDNA (Quant-iT PicoGreen dsDNA Assay Kit, P7589, Invitrogen, Waltham, MS, USA). All DAMPs were measured by making a 1:1 dilution of serum in assay diluent, except for α-defensin, which was diluted 1:20. Fibrinogen levels were determined using the fibrinogen functional turbidimetric assay [31].

Statistical analysis

Continuous data are reported as mean±SD or median (interquartile range), where appropriate. To compare groups a *t*-test, Mann–Whitney U-test or Chi-square test was used. p-values below 0.05 were considered statistically significant. The strength of the associations between the DAMPs and the inflammation marker C-reactive protein (CRP) were assessed using Spearman's correlation coefficient (ρ).

Since no "normal" ranges are known for these DAMPs, maximally selected rank statistics were used to define a cut-off value that maximises the separation of the survival curves between the two formed groups in which the smallest group contained at least 10% of the observations [32]. In the case of Hsp70, <10%

of the patients had a serum level above 0, therefore 0 was used as the cut-off value. Next, a Cox proportional hazards model was used to evaluate the impact of high or low DAMP levels, based on the previously determined cut-offs, on survival time, adjusted for age, sex, severity of airway obstruction (forced expiratory volume in 1 s (FEV_1) z-score), severity of hyperinflation (inspiratory capacity to total lung capacity (IC/TLC) ratio), body mass index (BMI) and whether the patient received a bronchoscopic lung volume reduction (BLVR) treatment.

Lastly, for DAMPs that were found to significantly affect 4-year survival, we compared baseline characteristics, including pulmonary function and quantitative computed tomography outcomes, between the two DAMP groups. All statistical analyses were performed using R statistical software (version 4.2.3, Vienna, Austria).

Results

Study population

A total of 1030 patients were enrolled in the Groningen severe COPD cohort. Additional serum was available and at least one of the DAMPs of interest was measured in 949 (92%) of these patients, who were included in the current analyses. The characteristics of the study population are shown in table 1. The study population consisted predominantly of females (66%) and former smokers (98%), and the majority had severe airflow obstruction (61% COPD Global Initiative for Chronic Obstructive Lung Disease (GOLD) severity stage IV).

Damage-associated molecular patterns

In 637 (67%) of the included patients all DAMPs of interest were measured. In the other 312 (33%) patients at least one of the DAMPs was measured. The serum levels of all DAMPs are shown in table 2. Between DAMPs only negligible or weak associations were found, the strongest being between HMGB1 and dsDNA (ρ=0.290) and between α-defensin and galectin-9 (ρ=0.210).

TABLE 1 Demographic and patient characteristics

	n	Overall[#]	Alive[¶]	Dead[+]	p-value
Age years	949	62±7	61±7	64±8	<0.001
Sex, male	949	318 (34)	233 (33)	85 (36)	0.451
BMI kg·m^{-2}	948	24±4	24±4	23±4	<0.001
Smoking status	944				0.014
Former smoker		921 (97.6)	694 (98.3)	225 (95.7)	
Current smoker		20 (2.1)	12 (1.7)	8 (3.4)	
Never-smoker		3 (0.3)	0 (0)	2 (0.9)	
Pack-years	919	40 (30–50)	40 (30–48)	40 (29–50)	0.418
GOLD severity stage	940				<0.001
I		1 (0.1)	1 (0.1)	0 (0)	
II		30 (3.2)	24 (3.4)	6 (2.6)	
III		333 (35.4)	279 (39.6)	54 (23.0)	
IV		576 (61.3)	401 (56.9)	175 (74.5)	
SGRQ, total score	908	58.7±13.6	57.3±13.7	62.8±12.4	<0.001
FEV_1 % predicted	940	27 (22–34)	28 (23–35)	24 (20–30)	<0.001
FEV_1 z-score	940	−4.39±0.70	−4.37±0.70	−4.46±0.7	0.109
RV % predicted	933	243±56	238±52	260±64	<0.001
RV z-score	933	3.81±1.16	3.69±1.10	4.15±1.28	<0.001
RV/TLC ratio %	934	61±9	60±8	65±9	<0.001
IC/TLC ratio %	933	24 (19–28)	25 (20–29)	20 (16–24)	<0.001
D_{LCO} % predicted	442	35 (28–43)	36 (30–44)	28 (25–35)	<0.001
D_{LCO} z-score	442	−5.82±1.61	−5.63±1.58	−6.55±1.49	<0.001

Data are expressed as mean±SD, median (interquartile range) or n (%), where appropriate. The classifications "alive" or "dead" are based on the vital status 4 years after inclusion. All pulmonary function measures are derived using the Global Lung Initiative reference equations. BMI: body mass index; GOLD: Global Initiative for Chronic Obstructive Lung Disease, SGRQ: St. George's Respiratory Questionnaire; FEV_1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity, IC: inspiratory capacity, D_{LCO}: diffusion capacity of the lungs for carbon monoxide. [#]: n=949; [¶]: n=711; [+]: n=238.

TABLE 2 Serum levels of danger-associated molecular patterns (DAMPs)

DAMPs	n	Overall[#]	Alive[¶]	Dead[+]	p-value
Fibrinogen g·L^{-1}	790	3.4 (3.0–3.9)	3.4 (3.0–3.9)	3.4 (2.9–4.0)	0.81
HMGB1 ng·mL^{-1}	774	0 (0–1.46)	0 (0–1.47)	0 (0–1.43)	0.30
α-Defensin ng·mL^{-1}	864	169 (132–271)	170 (134–270)	168 (125–279)	0.82
Hsp70 pg·mL^{-1}	862	0 (0–0)	0 (0–0)	0 (0–0)	0.79
S100A8 pg·mL^{-1}	862	0 (0–0)	0 (0–0)	0 (0–0)	0.27
Galectin-9 pg·mL^{-1}	862	1268 (893–1769)	1259 (884–1755)	1279 (919–1781)	0.77
dsDNA ng·mL^{-1}	842	95 (64–132)	93 (63–128)	98 (66–146)	0.12

Data are reported as median (interquartile range). p-values were determined using a Mann–Whitney U test. HMGB1: high mobility group box 1 protein; Hsp70: heat shock protein 70; S100A8: S100 calcium-binding protein A8; dsDNA: double-stranded deoxyribonucleic acid. [#]: n=949; [¶]: n=711; [+]: n=238.

A moderate association was found between fibrinogen and the inflammation marker CRP (ρ=0.520) (supplementary table E1 and figure E1). None of the other DAMPs were associated with CRP.

Survival

4 years after inclusion, 711 (75%) patients were still alive. As shown in table 1, patients who died during the 4-year follow-up period were significantly older, had significantly more airflow obstruction and hyperinflation. Comparing the DAMP levels between the group that was still alive and the group that died did not show significant differences (table 2).

The cut-offs resulting from the maximally selected ranks statistics for the DAMPs and the hazard ratios and 95% confidence intervals resulting from the adjusted Cox proportional hazards model are shown in table 3, supplementary tables E2 and E3, and figure E2. DsDNA, based on a cut-off of 148 ng·mL^{-1}, showed a significant effect on survival time (hazard ratio 1.44, 95% confidence interval: 1.04–2.00) with patients in the group with a value above 148 ng·mL^{-1} having a shorter survival time (figure 1). None of the other assessed DAMPs showed a significant association with survival time.

Serum dsDNA levels

Next, it was assessed whether there were differences in characteristics between the group of patients with dsDNA ⩽148 ng·mL^{-1} and the group with dsDNA >148 ng·mL^{-1} (table 4). The group of patients with "high" dsDNA serum levels consisted of significantly more males, had a significantly lower FEV_1 % predicted, although there was no difference in FEV_1 z-score, and significantly higher emphysema scores.

Discussion

Over the past 15 years, there has been a growing body of evidence supporting the critical role of DAMPs in the pathophysiology of COPD [7, 33]. However, the current study represents the first investigation specifically addressing the impact of serum DAMP levels on the survival of COPD patients using a large

TABLE 3 Cox proportional hazards regression model of association between danger-associated molecular patterns (DAMPs) and 4-year survival

DAMPs	n	HR	95% CI	p-value
Fibrinogen >3.9 g·L^{-1}	781	0.98	0.71–1.36	0.91
HMGB1 >0 ng·mL^{-1}	758	1.20	0.89–1.61	0.23
α-Defensin >372 ng·mL^{-1}	846	1.18	0.81–1.70	0.38
Hsp70 >0 pg·mL^{-1}	844	0.86	0.46–1.59	0.63
S100A8 >11 pg·mL^{-1}	844	0.75	0.48–1.18	0.22
Galectin-9 >2131 pg·mL^{-1}	844	0.76	0.51–1.15	0.20
dsDNA >148 ng·mL^{-1}	826	1.44	1.04–2.00	**0.028**

The model is adjusted for sex, age at inclusion, FEV_1 z-score, IC/TLC ratio, receiving BLVR treatment and BMI. Data in bold denotes statistical significance. HR: hazard ratio; HMGB1: high mobility group box 1 protein, Hsp70: heat shock protein 70; S100A8: S100 calcium-binding protein A8, dsDNA: double-stranded deoxyribonucleic acid.

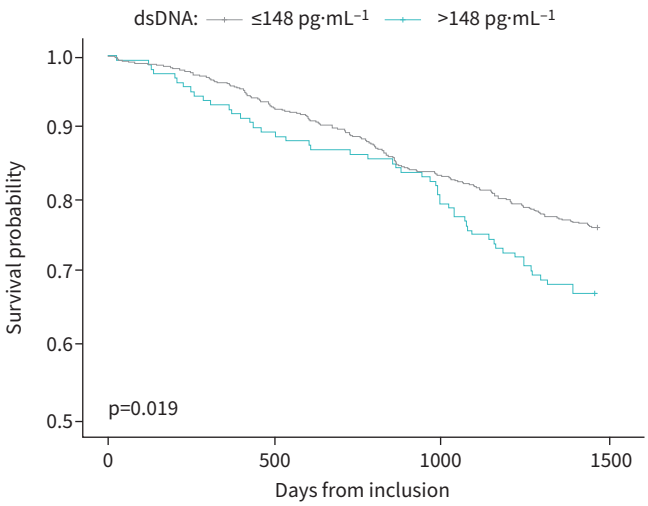

FIGURE 1 Kaplan–Meier curve comparing survival probability over time between patients with a double-stranded DNA (dsDNA) level ⩽148 pg·mL^{-1} and >148 pg·mL^{-1}. The reported p-value is unadjusted for any other variables.

clinical cohort. Within a panel of seven different DAMPs (fibrinogen, HMGB1, α-defensin, Hsp70, S100A8, galectin-9 and dsDNA), only elevated serum levels of dsDNA were significantly associated with reduced 4-year survival rates among patients with severe COPD.

Several studies reported elevated levels of DAMPs, such as HMGB1, Hsp70 and fibrinogen, in COPD patients when compared to "healthy" controls [26, 34, 35]. Additionally, prior research describes associations between elevated levels of fibrinogen and HMGB1 to COPD severity and the incidence of COPD exacerbations [26, 35]. A meta-analysis further identified fibrinogen, a widely assessed and more general pro-inflammatory factor, as a biomarker associated with premature death in COPD patients [36]. However, the results of our study do not support this previous finding, as we did not observe a significant association between an elevated serum level of fibrinogen and risk of mortality. A potential explanation for

TABLE 4 Comparison of formed double-stranded DNA groups

	n	dsDNA ⩽148 ng·mL^{-1}[#]	dsDNA >148 ng·mL^{-1}[¶]	p-value
Age years	842	62±7	61±8	0.21
Sex, male	842	205 (30)	78 (49)	**<0.001**
BMI kg·m^{-2}	841	24±4	24±4	0.20
Pack-years	817	40 (30–49)	40 (30–50)	0.52
SGRQ, total score	813	58 (50–67)	59 (51–68)	0.24
FEV_1 % predicted	834	28 (22–34)	25 (21–32)	**0.006**
FEV_1 z-score	834	−4.4±0.7	−4.5±0.7	0.081
RV % predicted	828	241±57	248±56	0.21
RV z-score	828	3.8±1.2	3.9±1.2	0.32
RV/TLC ratio %	828	61±9	61±9	0.61
IC/TLC ratio %	827	24 (19–28)	23 (19–28)	0.32
D_{LCO} % predicted	467	35 (28–43)	34 (26–42)	0.31
D_{LCO} z-score	467	−5.8 (−6.9– −4.7)	−5.9 (−7.3– −4.7)	0.33
Lung mean density HU	782	−880 (−892– −866)	−879 (−892– −866)	0.82
Emphysema score at −950 HU %	782	37 (31–42)	40 (35–44)	**<0.001**
Received BLVR, no	837	476 (70)	107 (68)	0.72

Data are presented as mean±SD, median (interquartile range) or n (%). Data in bold denotes statistical significance. dsDNA: double-stranded deoxyribonucleic acid; BMI: body mass index; SGRQ: St. George's Respiratory Questionnaire; FEV_1: forced expiratory volume in 1 s; RV: residual volume; TLC: total lung capacity; IC: inspiratory capacity; D_{LCO}: diffusion capacity of the lungs for carbon monoxide; HU: Hounsfield units; BLVR: bronchoscopic lung volume reduction. [#]: n=682; [¶]: n=160.

this difference could be the stable respiratory condition of our study population, with no acute exacerbations or respiratory infections at the time of visit, considering a previous study indicated that fibrinogen levels tend to rise during acute exacerbations of COPD which in itself is associated with a high risk of mortality [35]. Furthermore, in our study population >95% of patients had severe COPD (GOLD stage III or IV), whereas previous studies included a higher proportion of patients with less severe airflow obstruction [37–39]. While one of these previous studies found an even stronger association between elevated fibrinogen levels and mortality in patients with COPD GOLD stage III or IV as compared to COPD GOLD stage II patients, it should be noted that only 3% of the study's patients fell into the GOLD III–IV category [38].

Our study indicates a significant association between an elevated serum level of dsDNA and an increased risk of death in patients with severe COPD, even after adjusting for established survival-related factors including sex, age, BMI, severity of airflow obstruction, severity of hyperinflation and whether the patients underwent a BLVR treatment [40–42]. Under physiological conditions, dsDNA resides in the cell nucleus but can be released passively during type III cell death, such as necrosis, or actively secreted as part of exosomes [43]. Once released into the extracellular space, dsDNA can be recognised by specific receptors, such as toll-like receptor 9, of the innate immune system, initiating cytokine release and potentially triggering pyroptosis, an inflammatory and lytic form of cell death [44, 45]. Circulating levels of cell-free DNA, including dsDNA and mitochondrial DNA, have been linked to the development and/or progression of various conditions such as autoimmune diseases, cancer, chronic kidney disease, chronic organ rejection and myocardial infarction [44, 46–50]. Our current study suggests that an elevated serum level of dsDNA might also have a potential role in identifying COPD patients at higher risk of death among other already known predictors.

The observed association between elevated serum levels of dsDNA and survival in our study may be attributed to a genetic predisposition to develop more severe COPD in response to cigarette smoke exposure. Previous studies have demonstrated that exposure to cigarette smoke induces the release of dsDNA from both lung epithelial cells and neutrophils and that the extent of dsDNA release is linked to the genetic susceptibility of cigarette smoke-induced neutrophilia in mice [8, 14, 15, 21, 51]. This hypothesis might even be supported by the finding that we found significantly more airflow obstruction and emphysematous destruction in the group with elevated levels of circulating dsDNA. However, it is crucial to acknowledge that these differences, although statistically significant, were relatively small from a clinical perspective.

The cohort used in our study consists predominantly of patients with severe COPD (GOLD stage III or IV) with an emphysematous phenotype, and former smokers. This raises the possibility that certain DAMPs that are more associated with survival in COPD patients with predominantly airway disease or with less advanced stages of airflow limitation are not picked up in our study. Additionally, given the known release of DAMPs upon cigarette smoke exposure, the lack of active smokers in our study population might have obscured our outcomes. Furthermore, we were unable to investigate whether serum DAMP levels were persistently increased, because serum samples were only obtained during a single visit. Future studies should be performed assessing the circulating dsDNA levels over time in relation to survival rates. It should also be noted that certain well-established predictors of survival in COPD patients, such as the 6-min walk distance or chronic hypercapnic respiratory failure, were not included in our analysis due to the unavailability of these data. Lastly, most DAMPs have only been assessed in experimental research conditions, so the "normal ranges" are unknown for most DAMPs, except fibrinogen. Consequently, the cut-off value to differentiate elevated DAMP levels was based on statistical methods which may lack clinical significance.

In conclusion, in a clinical cohort of 949 patients with severe COPD who were referred to potentially receive a BLVR treatment, elevated serum levels of dsDNA were associated with a higher risk of death, independent of sex, age, BMI, severity of airflow obstruction, severity of hyperinflation and whether the patients underwent a BLVR treatment. This study further illustrates the potential role of circulating DAMPs, such as dsDNA, in the progression of COPD. Future studies are needed to further unravel the associations between levels of DAMPs and survival and to investigate the clinical relevancy of using DAMPs as prognostic biomarkers for COPD survival.

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